

BACTERIAL HOSTS FOR RECOMBINANT PROTEIN EXPRESSION

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 63/209,239 filed June 10, 2021, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Microbial host cell expression systems are used for production of recombinant proteins with varying degrees of success. Recombinant protein degradation and low yield remain challenges. Alterations of the host cell genome to optimize the production of high-quality protein often result in frustratingly low host cell growth and production yields. Recombinant host cells that achieve both high quality and high yield of recombinant proteins remain needed.

SUMMARY OF THE INVENTION

[0003] The present invention provides recombinant bacterial host cells and methods for their use to produce high quality recombinant proteins at high yield. In particular, the compositions and methods provided by the invention are useful for producing recombinant proteins that are sensitive to proteolysis, including antibodies and antibody fragments. The invention includes recombinant gram-negative bacterial host cells comprising combinations of genetic modifications that result in reduced degradation of a recombinant protein while allowing growth to high cell density. In some embodiments, a recombinant gram-negative bacterial host cell of the present invention is deficient in first protease activity and a second protease activity, wherein the first protease activity is a tail-specific protease activity and the second protease activity is a murein DD-endopeptidase activity. In some embodiments, the invention relates to a recombinant gram-negative bacterial host cell that is deficient in tail-specific protease activity and a murein DD-endopeptidase activity, wherein the host cell produces high quality, undegraded, recombinant protein, and grows to a high cell density. In some embodiments, the murein DD-endopeptidase activity is a MepM activity. In some embodiments, the gram-negative bacterial host cell is not deficient in a MepS activity. In some embodiments, the recombinant gram-negative bacterial host cell deficient in a first protease activity and a second protease activity, wherein the first protease activity is a tail-specific protease activity, and the second protease activity is a MepM murein DD-endopeptidase activity, is further deficient in at least one additional protease activity, at least one autolytic factor activity, or both. In some embodiments, the at least one additional protease activity is a serralyisin precursor activity. The deficiency in a protein activity, e.g., the first protease, the second protease, an additional protease, or an autolytic factor, can result from a

mutation in one or more gene. The invention further provides methods for producing intact, soluble, and/or active recombinant proteins of interest at high yield using the inventive recombinant gram-negative bacterial host cells. In some embodiments, the recombinant protein of interest is a Fab'. In some embodiments, the Fab' binds to TNF- α .

[0004] The present invention includes a recombinant gram-negative bacterial host cell for recombinant protein expression, wherein the host cell is: (a) deficient in a first protease activity, wherein the first protease activity is tail-specific protease activity, wherein the deficient first protease activity results from a mutation in at least one gene encoding a tail-specific protease; (b) deficient in a second protease activity, wherein the second protease activity is murein DD-endopeptidase activity, wherein the deficient second protease activity results from a mutation in at least one gene encoding a murein DD-endopeptidase. In some embodiments, the recombinant gram-negative bacterial host cell further: (c) is deficient in at least one additional protease activity, wherein the deficient additional protease activity results from a mutation in at least one gene encoding an additional protease, wherein the additional protease is different from the proteases of (a) and (b); (d) is deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor; (e) overexpresses one or more inactivated protease; (f) overexpresses one or more folding modulator; or (g) any combination of (c), (d), (e) and (f). In some embodiments, the deficient tail-specific protease activity results from a mutation in a gene encoding one or more of: (i) a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33, a homologue of SEQ ID NO: 33, or a Prc1 tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 33; (ii) a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35, a homologue of SEQ ID NO: 35, or a Prc2 tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 35; or (iii) a Tsp tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 71, a homologue of SEQ ID NO: 71, or an Tsp tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 71. In some embodiments, the deficient murein DD-endopeptidase activity results from a mutation in a gene encoding one or more of: (i) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 1, a homologue of SEQ ID NO: 1, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 1; (ii) a MepM murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 63, a homologue of SEQ ID NO: 63, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 63; (iii) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 65, a homologue of SEQ ID NO: 65, or a murein DD-

endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 65; and (iv) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 66, a homologue of SEQ ID NO: 66, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 66. In some embodiments, the host cell of (c) is deficient in 1 to 10 different additional protease activities; the host cell of (d) is deficient in 1-5 different autolytic factor activities; the host cell of (e) overexpresses 1 to 10 different inactivated proteases, wherein each inactivated protease is different; the host cell of (f) overexpresses 1-10 different folding modulators, or any combination thereof. In some embodiments, the one or more deficient additional protease activity of (c) results from a mutation of at least one gene encoding an additional protease independently selected from: a serralyisin precursor, a membrane-localized protease, a murein L,D transpeptidase, a hemolysin precursor, a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor, a periplasmic serine endoprotease, an AAA+ family proteolytic machine, and a murein DD-endopeptidase different from that of (a); the one or more deficient autolytic factor activity of (d) results from a mutation of at least one gene encoding an autolytic factor independently selected from: an S-type pyocin, a linear gramicidin synthase subunit D, a hemolysin precursor, a leukotoxin, and a porin; the one or more inactivated protease of (e) is a mutant periplasmic serine endoprotease; and the one or more folding modulator of (f) is a disulfide isomerase. In some embodiments, the serralyisin precursor is selected from: a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 47; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 47; a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 47; the membrane-localized protease is an HtpX having the amino acid sequence set forth as SEQ ID NO: 39, a homologue of the HtpX having the amino acid sequence set forth as SEQ ID NO: 39, or an HtpX related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 39; the murein L,D transpeptidase is a murein L,D transpeptidase having the amino acid sequence set forth as SEQ ID NO: 41, a homologue of the murein L,D transpeptidase having the amino acid sequence set forth as SEQ ID NO: 41, or a murein L,D transpeptidase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 41; the hemolysin precursor is a hemolysin precursor having the amino acid sequence set forth as SEQ ID NO: 43, a homologue of the

hemolysin precursor having the amino acid sequence set forth as SEQ ID NO: 43, or a hemolysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 43; the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor is a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45, a homologue of the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45, or a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 45; the periplasmic serine endoprotease is selected from: a DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a homologue of the DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a DegP2 related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 31; a DegP having the amino acid sequence set forth as SEQ ID NO: 69; a homologue of the DegP having the amino acid sequence set forth as SEQ ID NO: 69; a DegP related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 69; a DegP having the amino acid sequence set forth as SEQ ID NO: 62; a homologue of the DegP having the amino acid sequence set forth as SEQ ID NO: 62; and a DegP related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 62; the AAA+ family proteolytic machine comprises an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38; the murein DD-endopeptidase is selected from: a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 3; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 3; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 3; a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64; or a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 64; a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67; a MepM2-related protein having at least 60% similarity or at least

60% identity to the amino acid sequence set forth as SEQ ID NO: 67; a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68; and a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 68; the S-type Pyocin is an S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49, a homologue of the S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49, or an S-type Pyocin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 49; the linear gramicidin synthase is a linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51, a homologue of the linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51, or a linear gramicidin synthase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 51; the leukotoxin is a leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53, a homologue of the leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53, or a leukotoxin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 53; the ShlB hemolysin transporter is an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55, a homologue of the an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55, or an ShlB hemolysin transporter related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 55; each of the one or more overexpressed inactivated proteases is independently selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2-related protein comprising an amino acid substitution or disruption of a DegP2 having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA-related protein comprising an amino acid substitution

or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of an amino acid at a position corresponding to any one of 131 (His), 134 (Asp) and 236 (Ser) (SEQ ID NO: 62, referring to numbering including leader sequence 1-26), or respective positions 105, 108, and 210, when excluding the leader sequence; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr S210A; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr H105R; and an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of any one or more amino acid at a position corresponding to any one of: 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234 of SEQ ID NO: 31; and each of the one or more folding modulators is independently selected from: a disulfide bond isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 76-81; a homologue of a disulfide bond isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 76-81; a disulfide bond isomerase-related protein having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as any one of SEQ ID NOS: 76-81; a protein disulfide isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 27 and 82-98; a homologue of a protein disulfide isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 27 and 82-98; and a protein disulfide isomerase-related protein having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NOS: 27 and 82-98.

[0005] In some embodiments, the mutation is in a coding sequence or noncoding sequence of the corresponding gene, and the mutation is independently selected from: (i) a complete gene deletion, (ii) a partial gene deletion, (iii) a missense mutation, (iv) a nonsense mutation, (v) a frameshift mutation, (vi) an insertion, and (vii) any combination of (ii), (iii), (iv), (v) and (vi). In some embodiments, the missense mutation of (iii) results in a conservative or non-conservative amino acid substitution. In some embodiments, the noncoding sequence is a regulatory sequence. In some embodiments, the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is the activity of: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 protease amino acid sequence set forth as SEQ ID NO: 5. In some embodiments, the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is: a MepS2 having the

amino acid sequence set forth as SEQ ID NO: 7; a homologue of the MepS2 having the amino acid sequence set forth as SEQ ID NO: 7; or a MepS2 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS2 protease amino acid sequence set forth as SEQ ID NO: 7. In some embodiments, the gram-negative bacterial host cell is a *Pseudomonad*. In some embodiments, the gram-negative bacterial host cell is a *Pseudomonad*, and the deficient first protease activity results from a mutation of a coding sequence and/or noncoding sequence of a gene encoding *P. fluorescens* Prc1 and/or a mutation of a coding sequence and/or noncoding sequence of a gene encoding *P. fluorescens* Prc2. In some embodiments, the second protease activity is deficient due to a mutation that results in a conservative or non-conservative substitution in an active site amino acid or an allosteric site amino acid of a protease having the second protease activity. In some embodiments, the deficient second protease activity results from at least one mutation of the second protease gene, wherein the mutation results in a disruption of the amino acid sequence at a position corresponding to: (i) any one or more of residues 134 to 145 of SEQ ID NO: 1; (ii) any one or more of residues 319 to 411 of SEQ ID NO: 1; (iii) one or more of residues any 361 to 378 of SEQ ID NO: 1; (iv) any one or more residue selected from 248, 319, 330, 332, 334, 337, 378, 410, and 411 of SEQ ID NO: 1; or any combination of (i), (ii), (iii), and (iv). In some embodiments, the bacterial host cell is *Pseudomonas fluorescens*, and the deficient second protease activity results from a gene mutation that results in an amino acid substitution of SEQ ID NO: 1 selected from: Y248stop, G332S, D334N, A337T, H411Y, P410L, and any conservative or non-conservative amino acid substitution of any one of R319, H330, D334, H378, and H411.

In some embodiments, the recombinant gram-negative bacterial host cell is capable of high-density cell growth in culture. In some embodiments, the high-density cell growth in culture comprises growth to OD575 of about 80 to about 300. In some embodiments, the high-density cell growth in culture is increased in comparison to a control cell by about 2-fold to about 15-fold. In some embodiments, the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from: (i) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional; (ii) a recombinant gram-negative bacterial host cell deficient in the first protease activity, the second protease activity, and an additional protease activity as recited in 2(c) hereinabove, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity of 2(c) that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and (iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the

second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell. In some embodiments, the additional protease activity of 2(c) is an activity of a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9.

[0006] In some embodiments, the recombinant gram-negative bacterial host cell further comprises at least one expression construct, each expression construct comprising at least one nucleic acid sequence encoding a recombinant protein of interest. In some embodiments, the recombinant protein of interest is native or heterologous to the recombinant gram-negative bacterial host cell. In some embodiments, the recombinant protein of interest is selected from: an antibody, antibody fragment, or derivative of an antibody or antibody fragment; an antibody-based drug, a non-antibody binding protein (e.g., an antibody mimetic, including, but not limited to, an alphabody, an iBody, an affibody, an affilin, an affitin, or an anticalin), a reagent protein; a vaccine antigen; a therapeutic protein or enzyme; non-natural protein; a pathogen protein or derivative thereof; a microbial toxin, a lipoprotein; an extracellular receptor or ligand; a protease; a kinase; a blood protein; a chemokine; a cytokine; a bone morphogenic protein; an anticoagulant; a blood factor; a bone morphogenetic protein; an engineered protein scaffold; an enzyme, e.g., a biocatalytic enzyme; a growth factor; an interferon; an interleukin; a thrombolytic agent; a hormone; and a TGF-beta family member protein. In some embodiments, the recombinant protein of interest is human, murine, rat, rabbit, guinea pig, camelid, shark, avian, yeast, fungal, gram-negative bacterial, or gram-positive bacterial. In some embodiments, the antibody, antibody fragment, or derivative thereof is selected from: a monoclonal antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; modified antibody, a bispecific antibody, a chimeric antibody; a diabody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a tribody; an intrabody; a nanobody; a small modular immunopharmaceutical (SMIP); an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody, an avian antibody (e.g., a chicken antibody), a VHH-containing antibody; a F(ab); a F(ab)'; F(ab)'₂; scFv; an Fc fragment generated from the

heavy chain constant region of an antibody; a reduced IgG fragment (e.g., generated by reducing the hinge region disulfide bonds of IgG); an Fc fusion protein (e.g., comprising the Fc domain of IgG fused together with a protein or peptide of interest); a domain antibody; a VL; a VNAR; a VH; and a VHH. In some embodiments, the VHH-containing antibody is a VHH concatenated antibody. In some embodiments, the antibody, antibody fragment, or derivative thereof, binds to a target selected from: a cytokine; a chemokine; a drug; a cell-surface protein, e.g., a receptor, cell-surface marker, pathogen surface-protein, etc.; a growth factor; a growth factor receptor; immune checkpoint molecule, and a blood factor. In some embodiments, the antibody, antibody fragment, or derivative thereof is a Fab'. In some embodiments, the Fab' binds to a target selected from: Carcinoembryonic antigen (CEA); CD22; fibrin II, beta chain; TNF-alpha; and NCA-90 (granulocyte antigen). In some embodiments, the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least one nucleic acid sequence encoding a heavy chain, at least one nucleic acid sequence encoding a light chain, or both, wherein the heavy chain is full-length or a heavy chain fragment, and the light chain is full-length or a light chain fragment. In some embodiments, the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least two nucleic acid sequences, each encoding a heavy chain. In some embodiments, the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from the same mRNA transcript. In some embodiments, the at least one expression construct encoding the antibody, an antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from different mRNA transcripts. In some embodiments, each heavy chain-encoding nucleic acid sequence and each light chain-encoding nucleic acid sequence is individually operably linked to an independently selected nucleic acid sequence encoding a periplasmic secretion signal. In some embodiments, the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NO: 11, 13, 15, or 17. In some embodiments, the expression construct comprises: a nucleic acid sequence encoding an antibody heavy chain, operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NOS: 11, 13, 15, or 17; a nucleic acid sequence encoding a light chain, operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NOS: 11, 13, 15, or 17; or both. In some embodiments, the antibody, antibody fragment, or derivative thereof is humanized. In some embodiments, the Fab' is certolizumab. In some embodiments,

the Fab' heavy chain has the amino acid sequence set forth as SEQ ID NO: 21, and the Fab' light chain has the amino acid sequence set forth as SEQ ID NO: 23. In some embodiments, the nucleic acid sequence encoding the heavy chain is operably linked to a nucleic acid sequence encoding a secretion leader having the amino acid sequence set forth as SEQ ID NO: 11, and the nucleic acid sequence encoding the light chain is operably linked to a nucleic acid sequence encoding a secretion leader having the amino acid sequence set forth as SEQ ID NO: 13.

[0007] In some embodiments, the recombinant gram-negative bacterial host cell is deficient in: (i) the first protease activity; (ii) the second protease activity; (iii) the activity of a serralsin precursor having the amino acid sequence set forth as SEQ ID NO: 9, a homologue of the serralsin precursor having the amino acid sequence set forth as SEQ ID NO: 9, or a serralsin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; (iv) an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and (v) an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38. In some embodiments, the host cell further overexpresses an exogenous inactivated DegP, wherein the inactivated DegP is selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 derived from the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP2 derived from a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from a DegP2 having at least 60% similarity or at least 60% identity to amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from a DegP2 having at least 60% similarity or at least 60% identity to amino acid sequence set forth as SEQ ID NO: 62; and each of the proteases having the amino acid sequence set forth as SEQ ID NO: 31 comprising a conservative or nonconservative amino acid substitution or disruption of any one or more of residues 116, 146, 219, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234. In some embodiments, the recombinant gram-negative bacterial host cell overexpresses an exogenous disulfide isomerase selected from any one of: a disulfide isomerase having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NO: 27, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73, and a homologue of a disulfide

isomerase having the amino acid sequence set forth as SEQ ID NO: 27, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, and 73.

[0008] In some embodiments, the recombinant gram-negative bacterial host cell is selected from: a *Pseudomonad* host cell; an *E. coli* host cell; and a *Vibrio* host cell. In some embodiments, the host cell is a gram-positive host cell, e.g., a *Bacillus* host cell. In some embodiments, the *Pseudomonad* host cell is a *Pseudomonas* host cell. In some embodiments, the *Pseudomonas* host cell is *P. fluorescens*, *P. putida*, or *P. aeruginosa*. In some embodiments, the recombinant gram-negative bacterial host cell is: (i) *lsc::lacIQ1*; (ii) *Prc1*-; (ii) *Prc2*-; (iii) *HslU*-; (iv) *HslV*-; (v) *MepM1*-; (vi) *PyrF*-; and (vii) deficient in a serralyisin precursor that is: a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; wherein the serralyisin precursor deficiency results from a mutation in a gene encoding the serralyisin precursor. In some embodiments, the recombinant gram-negative bacterial host cell is *P. fluorescens*, the *Prc1* has the amino acid sequence set forth in SEQ ID NO: 33, the *Prc2* has the amino acid sequence set forth in (SEQ ID NO: 35), the *HslU* has the amino acid sequence set forth in (SEQ ID NO: 37), the *HslV* has the amino acid sequence set forth in (SEQ ID NO: 38), the *MepM1* has the amino acid sequence set forth in (SEQ ID NO: 1), and the serralyisin precursor has the amino acid sequence set forth as SEQ ID NO: 9. In some embodiments, the host cell further comprises an expression vector comprising a nucleic acid sequence encoding DegP2 S219A (SEQ ID NO: 29). In some embodiments, the recombinant gram-negative bacterial host cell further comprises an expression vector comprising a nucleic acid sequence encoding disulfide isomerase PDIA6 (SEQ ID NO: 27). In some embodiments, the host cell further comprises an expression vector encoding a recombinant protein. In some embodiments, the expression vector encodes a Fab'. In some embodiments, the expression vector comprising the nucleic acid sequence encoding DegP2 S219A or disulfide isomerase PDIA6 further comprises a nucleic acid sequence encoding the Fab'. In some embodiments, the Fab' heavy chain is encoded by SEQ ID NO: 21, and the Fab' light chain is encoded by SEQ ID NO: 23. In some embodiments, the recombinant gram-negative bacterial host cell is a *Pseudomonad* having the genotype of strain STR94975, STR94976, or STR94977. In some embodiments, the recombinant gram-negative bacterial host cell, further comprises the expression construct or constructs comprised by a plasmid of STR94975, STR94976, or STR94977, for use in producing a recombinant anti-TNF-alpha Fab'. In some embodiments, the gram-negative bacterial host cell is not *E. coli*.

[0009] The present invention further includes a method for producing a recombinant protein of interest comprising: (a) recovering the recombinant protein of interest from a recombinant gram-negative bacterial host cell of the invention cultured under suitable fermentation conditions, wherein the recombinant gram-negative host cell is transformed with a plasmid comprising a nucleic acid encoding the recombinant protein of interest. In some embodiments, transcription of the nucleic acid sequence encoding the recombinant protein of interest is regulated by an inducible promoter. In some embodiments, the inducible promoter is selected from: a tac promoter, a mannitol promoter, a Pben, a T7 promoter, a lac promoter, a T5 promoter, a xylose promoter, and an arabinose promoter. In some embodiments, the recombinant gram-negative bacterial host cell can grow to high cell density. In some embodiments, the high cell density comprises an OD575 of about 80 to about 300. In some embodiments, the suitable fermentation conditions comprise induction of the inducible promoter at: an OD575 of about 80 to about 160, a culture pH of about 5.8 to about 7.0, a temperature of about 28-33 deg C, fed-batch, and a titer range of about 0.2 to about 5 g/L. In some embodiments, the inducible promoter is induced by IPTG, and wherein the IPTG is added to a final concentration of about 0.08 – 0.3mM. In some embodiments, the IPTG is added to a final concentration of about 0.2 mM. In some embodiments, induction is carried out at a culture pH of about 6.0 to about 6.5. In some embodiments, induction is carried out at a temperature of about 28-33 deg C. In some embodiments, induction is carried out at a temperature of about 32 deg C. In some embodiments, the recombinant gram-negative bacterial host cell grows to a cell density that is increased in comparison to a control cell grown under the same fermentation conditions. In some embodiments, the increase in cell density is about 2-fold to about 15-fold. In some embodiments, the method further comprises: (b) measuring the yield of intact, soluble, and/or active, recombinant protein of interest recovered from the recombinant gram-negative bacterial host cell. In some embodiments, the measured yield of intact, soluble, and/or active, recombinant protein is about 0.1 to about 10 g/L. In some embodiments, the method further comprises: (c) measuring the yield of recombinant protein of interest recovered from a control cell that is intact, soluble, active, or a combination thereof. In some embodiments, the method further comprises (d) comparing the yield measured in step (b) to the yield measured in step (c). In some embodiments, the yield measured in step (b) is about 2-fold to about 100-fold higher than that measured in step (c). In some embodiments, the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from: (i) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional; (ii) a recombinant gram-negative bacterial host cell

deficient in the first protease activity, the second protease activity, and an additional protease activity as recited in 2(a), and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity of 2(a) that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and (iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell. In some embodiments, the gram-negative bacterial host cell is not *E. coli*.

[0010] The invention also includes a recombinant polypeptide comprising: a secretion signal peptide operably linked to a heterologous protein or polypeptide of interest, wherein the secretion signal peptide has the amino acid sequence set forth in SEQ ID NO: 11. The protein or polypeptide of interest can be: an antibody, antibody fragment, or a derivative of an antibody or an antibody fragment; an enzyme; a cytokine; a chemokine; a growth factor; a fusion protein; and a vaccine antigen. In some embodiments, the antibody, antibody fragment, or a derivative of an antibody or antibody fragment is selected from: a monoclonal antibody; a full chain antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; a modified antibody; a variable-region only antibody fragment; a bispecific antibody, a chimeric antibody; a tribody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a dibody; an intrabody; a nanobody; a small modular immunopharmaceutical; an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody (VNAR); an avian antibody; a VHH; a VHH-containing antibody; a VHH concatemer; a F(ab); a F(ab)'; F(ab)'₂; an scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment; an Fc fusion protein; a domain antibody; a VL; and a VH. In some embodiments, the antibody, antibody fragment, or derivative of the antibody or antibody fragment is humanized. In some embodiments, the enzyme is a therapeutic enzyme. In some embodiments, the therapeutic enzyme is selected from: a peptidase; a lactase; an amylase; a PEP; a digestive enzyme; a uricase; a rhodanase; a urokinase; a streptokinase; a staphylokinase; a phenylase; a sacrosidase; a lysozyme; a chitinase; a ribonuclease; a glutaminase; an arginase; a vibrilase; a chondroitinase; a hyaluronidase; a galactosidase; a glucuronidase; a glucocerebrosidase; a thymidine

phosphorylase; a carbonic anhydrase; a uricase thiosulfate-cyanide; a sulfurtransferase; a phosphothioesterase; an alcohol oxidase; an alcohol dehydrogenase; an asparaginase; a glutamine synthase; an adenosine deaminase; bovine pegademase; alglucerase; dornase alpha; imiglucerase; sacrosidase; rasburicase; agalsidase beta; and nattokinase. In some embodiments, the fusion protein is selected from: an enzyme fusion protein; a protein A fusion protein; an albumin fusion protein; a thioredoxin fusion protein; a ubiquitin fusion protein; a streptavidin fusion protein; a maltose binding protein fusion protein; a chitin binding protein fusion protein; a SUMO fusion protein; and a glutathione-S-transferase fusion protein. In some embodiments, the polypeptide further comprises a linker. In some embodiments, the polypeptide further comprises a cleavage domain. In some embodiments, the secretion signal peptide directs expression of the protein or polypeptide of interest to the periplasm or the extracellular space of a prokaryotic host cell. In some embodiments, the protein or polypeptide of interest is expressed in the periplasm properly cleaved from the secretion signal peptide. In some embodiments, the secretion signal peptide directs expression of the protein or polypeptide of interest to the periplasm or the extracellular space of a prokaryotic host cell in properly cleaved form, soluble form, active form, or any combination thereof. A properly cleaved protein or polypeptide of interest may have an intact or substantially intact N-terminus. In some embodiments, the properly cleaved protein or polypeptide of interest having an intact or substantially intact N-terminus comprises the N-terminal methionine. In some embodiments, the properly cleaved protein or polypeptide of interest having an intact or substantially intact N-terminus does not comprise the N-terminal methionine. In some embodiments, the protein or polypeptide of interest requires a substantially intact N-terminus for substantial activity. In some embodiments, the protein or polypeptide of interest having a substantially intact N-terminus has about 90-100% of its activity when compared to the same protein or polypeptide of interest having an intact N-terminus. The prokaryotic host cell may be a gram-negative bacterium. The prokaryotic host cell may be a gram-positive bacterium. The gram-negative bacterium may be a *Pseudomonad*, *V. natriegens*, or *E. coli*. The gram-positive bacterium may be a *Corynebacterium* or a *Bacillus*. The invention includes an expression vector comprising a nucleic acid sequence encoding the recombinant polypeptide. The invention includes a prokaryotic host cell comprising an expression vector comprising a nucleic acid sequence encoding the recombinant polypeptide. In some embodiments, an expression vector and/or a nucleic acid construct encoding the recombinant polypeptide comprises a nucleic acid sequence that encodes the secretion signal peptide of SEQ ID NO: 11, operably linked to a nucleic acid sequence that encodes the heterologous protein or polypeptide of interest. In some embodiments, the secretion signal peptide amino acid sequence is encoded by a nucleic acid sequence having 85-100% sequence identity to SEQ ID NO: 12.

The prokaryotic host cell may be a gram-negative bacterium. The prokaryotic host may be a gram-positive bacterium. The gram-negative bacterium may be a *Pseudomonad*, *V. natrieigens*, or *E. coli*. The gram-positive bacterium may be a *Corynebacterium* or a *Bacillus*. In some embodiments, the nucleic acid sequence encoding the recombinant polypeptide is optimized for expression in the prokaryotic host cell. The invention also includes the use of a recombinant polypeptide, an expression vector, or a prokaryotic host cell, as described herein, for expressing a protein or polypeptide of interest in the periplasm or the extracellular space of a prokaryotic host cell.

[0011] The invention further includes a method of producing a protein or polypeptide of interest in a prokaryotic host cell, the method comprising: producing the protein or polypeptide of interest in the periplasm of a prokaryotic host cell cultured in a cell culture growth medium, wherein the prokaryotic host cell comprises an expression construct comprising a nucleic acid encoding a recombinant polypeptide comprising the protein or polypeptide of interest operably linked to a secretion signal peptide that directs expression of the protein or polypeptide of interest to the periplasm of the prokaryotic host cell, wherein the secretion signal peptide comprises the amino acid sequence set forth in SEQ ID NO: 11, and wherein the secretion signal peptide is not native to the protein or polypeptide of interest. The method may further comprise isolating the produced protein or polypeptide of interest. The protein or polypeptide of interest may be selected from: an antibody, antibody fragment, or a derivative of an antibody or an antibody fragment; an enzyme; a cytokine; a chemokine; a growth factor; a fusion protein; and a vaccine antigen. In some embodiments, the antibody, antibody fragment, or a derivative of an antibody or an antibody fragment is selected from: a monoclonal antibody; a full chain antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; a modified antibody; a variable-region only antibody fragment; a bispecific antibody, a chimeric antibody; a tribody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a dibody; an intrabody; a nanobody; a small modular immunopharmaceutical; an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody (VNAR); an avian antibody; a VHH; a VHH-containing antibody; a VHH concatemer; a F(ab); a F(ab)'; F(ab)'2; an scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment; an Fc fusion protein; a domain antibody; a VL; and a VH. In some embodiments, the antibody, antibody fragment, or derivative of the antibody or antibody fragment is humanized. In some embodiments, the enzyme is a therapeutic enzyme. In some embodiments, the therapeutic enzyme is selected from: a peptidase; a lactase; an amylase; a PEP; a digestive enzyme; a uricase; a rhodanase; a urokinase; a streptokinase; a staphylokinase; a phenylase; a sacrosidase; a

lysozyme; a chitinase; a ribonuclease; a glutaminase; an arginase; a vibrilase; a chondroitinase; a hyaluronidase; a galactosidase; a glucuronidase; a glucocerebrosidase; a thymidine phosphorylase; a carbonic anhydrase; a uricase thiosulfate-cyanide; a sulfurtransferase; a phosphothioesterase; an alcohol oxidase; an alcohol dehydrogenase; an asparaginase; a glutamine synthase; an adenosine deaminase; bovine pegademase; alglucerase; dornase alpha; imiglucerase; sacrosidase; rasburicase; agalsidase beta; and nattokinase. In some embodiments, the fusion protein is selected from: an enzyme fusion protein; a protein A fusion protein; an albumin fusion protein; a thioredoxin fusion protein; a ubiquitin fusion protein; a streptavidin fusion protein; a maltose binding protein fusion protein; a chitin binding protein fusion protein; a SUMO fusion protein; and a glutathione-S-transferase fusion protein. In some embodiments, the nucleic acid encodes a linker. In some embodiments, the linker comprises a cleavage domain. The prokaryotic host cell may be a gram-negative bacterium. The prokaryotic host cell may be a gram-positive bacterium. The gram-negative bacterium may be a *Pseudomonad*, *V. natriegens*, or *E. coli*. The gram-positive bacterium may be a *Corynebacterium* or a *Bacillus*.

INCORPORATION BY REFERENCE

[0012] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0014] Figure 1. Recombinant protein production in protease-deficient host cells at 0.5 mL scale. Nonreducing SDS-CGE analysis of Fab' protein produced by DC1032 and four other protease-deficient *P. fluorescens* strains tested is shown. The twelve lanes for each strain show, from left to right: far left lane - MW ladder with MW's 16, 20, 29, 48, and 68 kD; lanes numbered 1-10 – protein expressed using constructs 1-10, each encoding the Fab' heavy and light chain. Each of constructs 1-9 had a nucleic acid sequence encoding a different periplasmic secretion signal operably linked to the heavy chain gene and each had a nucleic acid sequence encoding the Azu secretion signal operably linked to the light chain gene. Construct 10 had the same heavy chain secretion signal as construct 3. Constructs 1-9 each co-expressed *P. fluorescens* derived DsbC, while construct 10 did not. Lane 12 (far right lane in each set) – null host strain (with a null expression plasmid). From left to right, the first 12 lanes show Fab'

expression in host strain DC1084; the second 12 lanes show Fab' protein expression in DC977; the third 12 lanes show Fab' protein expression in DC441; the fourth 12 lanes show Fab' protein expression in DC1032; and the fifth 12 lanes show Fab' protein expression in DC509. The arrow below the DC1032 lanes indicates the area of migration for the Fab' degradation products.

[0015] Figure 2. Growth of Prc null host strains at 2L scale under sub-optimal conditions.

Y-axis: OD575, X-axis: elapsed fermentation time at 36 deg C in hours. The gridlines mark 4-hour intervals, with the last timepoint taken at 62 hours.

[0016] Closed circles: STR36306 (Fab' expression plasmid; growth to a max OD575 of about 27)

[0017] Diamonds: STR94998 (DC1032 + null plasmid; growth to a max OD575 of about 50)

[0018] Triangles: STR94994 (DC1032 MepS1 deletion + p688-048; growth to a max OD575 of about 23)

[0019] Squares: STR94995 (DC1032 MepS2 deletion + p688-048; growth to a max OD575 of about 33)

[0020] Open circles: STR94996 (DC1032 MepS1 deletion and MepS2 deletion + p688-048; growth to a max OD575 of about 22).

[0021] Figures 3A and 3B. Prc1- and Prc2-deficient host strain growth at sub-optimal

conditions. 3A. Growth of DC954 colonies on 1X LB agar medium in the absence of NaCl and in the presence of uracil (to allow growth given the pyrF deletion) at 36 deg C. Colonies with background growth are visible. **3B.** The plate on the left shows growth of DC454 (no protease deletion) on 0.5X LB agar medium in the absence of NaCl, with uracil, at 36 deg C. Growth was slower than on 1X LB, but these growth conditions were not lethal. The plate on the right shows growth of DC954 on 0.5X LB agar medium in the absence of NaCl, with uracil, at 36 deg C. The adapted (evolved) cells are capable of growth.

[0022] Figure 4. Growth of evolved host strains at 2L scale. Y-axis: OD575, X-axis: fermentation time in hours. The gridlines mark 4-hour intervals, with the last timepoint taken at 74 hours (induction at 25.5 hours). Two cultures of each strain were grown, corresponding to the two curves for each strain #.

[0023] Triangles: PF1550, evolved from host DC954 to include MepM1 deficiency, resulting genotype $\Delta prc1$, $\Delta prc2$, MepM1(P410L), $\Delta pyrF$, $lsc::lacIQ1$ + p688-48 (Fab')

[0024] Open circles: STR36306, host DC1032 having genotype $\Delta prc1$, $\Delta prc2$, $\Delta hslUV$, $\Delta pyrF$, $lsc::lacIQ1$ + p688-48 (Fab')

[0025] Squares: PF1557 evolved having genotype $\Delta prc1$, $\Delta prc2$, MepM1(P410L), $\Delta pyrF$, $lsc::lacIQ1$ + pDOW1169 (empty expression vector)

[0026] Closed circles: DC432, host DC454 having genotype Δ pyrF + pDOW1169 (empty expression vector).

[0027] **Figure 5. Growth+ combinatorial MepM and MepS mutants.** Examples of growth by selected combinatorial mutants. From left to right, each of the following mutant strains (all Δ prc1, Δ prc2 except DC454 control; see **Table 6** for genotypes) was spotted on 0.5X LB + 250 ug/ml uracil agar medium in the absence of NaCl, and incubated for 48 hours at 36 deg C. In each row, five 10-fold serial dilutions were spotted, in descending concentration from the top to the bottom of the plate as oriented in the figure.

[0028] Column 1: DC1032 (Prc-)

[0029] Column 2: PF1559

[0030] Column 3: PF1588

[0031] Column 4: PF1560

[0032] Column 5: PF1590

[0033] Column 6: PF1572

[0034] Column 7: PF1577

[0035] Column 8: PF1573

[0036] Column 9: PF1575

[0037] Column 10: DC454 (Prc+)

[0038] **Figure 6. Light chain proteolysis in protease-deficient host strains.** STR87639, STR92557, STR92567, STR94974, and STR94976 were grown and harvested at the post-induction times (in hours) as shown on the y-axis, and the recombinant protein Capto-L enriched and analyzed by NR-SDS-CGE. The x-axis shows the percent of proteolyzed light chain species in each lane.

[0039] **Figure 7. Titer of recombinant Fab' produced by protease-deficient host strains under various fermentation conditions.** Fab' titer (Y-axis) from strains STR94974, STR94975, and STR94977 induced under varying pH and temperature conditions at the 2L fermenter scale was assessed by BLI measuring Fab' binding to TNF-alpha. Growth was performed at 2L bioreactor scale at 32 deg C, pH 6.5 and induced with 0.2 mM IPTG +/- 5g/L mannitol at OD575 at various pH and temperature values. The fermentation unit identifiers are shown along the X-axis. DG3_u1 (STR94974, 28 deg C and induced at pH 6.5), DG3_u2 (STR94974 25 deg C and pH6), DG3_u3 (STR94974 32 deg C and pH6), DG3_u4 (STR94974 25 deg C and pH 7), DG3_u5 (STR94975 28 deg C, pH 6.5, 5g/L mannitol), DG3_u6 (STR94975 25 deg C, pH 6.0, 5g/L mannitol), DG3_u7 (STR94975 32 deg C, pH 6.0 and 5g/L mannitol), DG3_u8 (STR94975 25 degC, pH 7.0), DG5_u1 (STR94977 28 deg C, pH6.5), DG5_u2 (STR94977 25 deg C, pH 6.0), DG5_u3 (STR94977, 32 deg C pH 6.0), DG5_u4

(STR94977 25 deg C, pH 7.0), DG5_u5 (STR94974 32deg C, pH 6.0), DG5_u6 (STR94975 32 deg C, PH 6.0, 5g/L mannitol), DG5_u7 (STR94975 25 deg C pH 7, 5g/L mannitol), DG3_u8 (STR94977 32 deg C, pH 6.0). Samples of whole cell broth at post-induction times 24, 48 and 72 hrs were processed and analyzed. At 72 hrs cells were separated from the media to assess Fab' titer in cell free broth (CFB).

[0040] Figures 8A and 8B. Growth comparisons of strain STR87639 with STR92557, STR92567, STR94974, and STR94976. Graphs comparing the growth of Fab' expression strains grown at 32 deg C and pH 6.5. Cultures were induced at elapsed fermentation time (EFT) 19.5 hours with 0.2mM IPTG and at 32 deg C, pH 6.0. OD575 is shown on the Y-axis and EFT on the X-axis. **8A.** Representative growth curves of the strains up to 24hrs post induction. **8B.** Growth curves of the strains up to 48 hrs post-induction. In both **8A** and **8B**:

[0041] Closed squares, solid line: STR87639 I24

[0042] Triangles, solid line: STR94974 #1

[0043] Triangles, dotted line: STR94974 #2

[0044] Triangles, dashed line: STR94974 #3

[0045] Circles, solid line: STR94976 I24

[0046] Circles, dotted line: STR94976 #2

[0047] Circles, dashed line: STR94976 #3

[0048] Open diamonds, solid line: STR92557 #1

[0049] Open diamonds, dotted line: STR92557 #2

[0050] Open diamonds, dashed line: STR92557 #3

[0051] Open squares, solid line: STR92567 I24.

[0052] Figure 9. Growth comparisons of strain STR87639 with STR92473, STR94994, STR94995, STR94996, and STR94998.

[0053] Graph compares growth of Prc-deficient Fab' expression strains STR87639, STR92473, STR94994, STR94995, and STR94996, and STR94998. Growth phase was conducted at 32 deg C, pH 6.5.

[0054] Closed circles, solid line: STR87639

[0055] Triangles, solid line: STR92473

[0056] Diamonds, solid line: STR94995

[0057] Circles, dashed line: STR94998

[0058] Open circles, solid line: STR94996

[0059] Squares, solid line: STR94994

[0060] Figure 10. Alignment showing murein DD-endopeptidase conserved amino acid residues. Conserved residues are in white lettering on dark background. Row 1 (top row) = P.

fluorescens MepM1 (SEQ ID NO: 1); Row 2 (second from top) = *E. coli* MepM (SEQ ID NO: 63, also known as YebA); Row 3 = *P. aeruginosa* MepM1 (SEQ ID NO: 66); Row 4 (bottom row): *P. putida* MepM1 (SEQ ID NO: 65).

DETAILED DESCRIPTION OF THE INVENTION

[0001] Provided herein are compositions and methods for producing high quality recombinant proteins at high yield.

[0002] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. In some embodiments of any of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of.” The phrase “consisting essentially of” is used herein to require the specified feature(s) as well as those which do not materially affect the character or function of the claimed invention. As used herein, the term “consisting” is used to indicate the presence of the recited feature alone. It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method or composition of the present disclosure, and vice versa. Furthermore, compositions of the present disclosure can be used to achieve methods of the present disclosure.

[0061] Reference in the specification to “embodiments,” “certain embodiments,” “preferred embodiments,” “specific embodiments,” “some embodiments,” “an embodiment,” “one embodiment” or “other embodiments” mean that a particular feature, structure, or characteristic described in connection with the embodiments is included in at least some embodiments, but not necessarily all embodiments, of the present disclosure.

[0062] Recombinant Protein Expression in Bacterial Host Cells

[0063] Recombinant proteins expressed in bacterial host cells are subject to degradation by any of several dozen host cell proteases. Degradation lowers protein quality and yield, often making production of useful quantities of proteolytically sensitive proteins impossible. Although the introduction of protease deficiencies in the host cell can reduce recombinant protein degradation, such deficiencies can prevent the host cell from growing to high density. Poor cell growth reduces recombinant protein yield, negating the advantage of the protease gene mutation. For example, as described herein, despite showing reduced degradation of recombinant proteins, bacteria deficient in tail-specific protease activity grow poorly.

[0064] The present invention relates to recombinant gram-negative bacterial host cells that are genetically engineered to produce high quality recombinant proteins without compromising cell growth. In some embodiments, the present invention relates to recombinant gram-negative bacterial host cells deficient in tail-specific protease activity, that are further modified to restore growth to high cell density. The growth inhibition resulting from deficient tail-specific protease activity is overcome by the introduction of one or more further genetic modifications resulting in deficiency of protease activity. Often more than one host cell protease activity causes unwanted recombinant protein degradation. To remedy this, the invention also provides host cells having additional protein deficiencies discovered to further enhance recombinant protein quality. Also provided are methods for use of the inventive host cells to produce high quality (e.g., active, soluble, and/or intact) recombinant proteins of interest, at high yield.

[0065] Host Cell Protein Deficiencies that Enhance Recombinant Protein Production

[0066] The present invention provides recombinant gram-negative bacterial host cells deficient in a first protease activity and a second protease activity. In some embodiments, a recombinant gram-negative bacterial host cell of the invention is: deficient in a first protease activity and a second protease activity, and further (a) is deficient in one or more additional protease activity; (b) is deficient in one or more autolytic factor activity; (c) overexpresses one or more inactivated protease; (d) overexpresses one or more chaperone or folding modulator protein; or (e) any combination of (a), (b), (c) and (d). In some embodiments, an additional protease, an autolytic factor, an inactivated protease, a chaperone or folding modulator, the first protease and the second protease, are each different. In some embodiments, an additional protease, an autolytic factor, an inactivated protease, or a chaperone or folding modulator, are the same as the first protease and/or the second protease. In some embodiments, the protease that is inactivated is the same as an additional protease. It is understood that the overexpressed inactivated protease of (c) and the overexpressed chaperone or folding modulator protein of (d) are different from a recombinant protein of interest to be overexpressed in the host cell. An overexpressed inactivated protease of (c) and/or an overexpressed chaperone or folding modulator protein of (d) can thus be co-overexpressed with a recombinant protein of interest.

[0067] In some embodiments, a recombinant gram-negative bacterial host cells is deficient in a first protease activity and a second protease activity, and deficient in one or more additional protease activity. In some embodiments, a recombinant gram-negative bacterial host cells is deficient in a first protease activity and a second protease activity, and deficient in one or more autolytic factor activity. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, and overexpresses one or more inactivated proteases. In some embodiments, a recombinant gram-negative bacterial host

cell is deficient in a first protease activity and a second protease activity, and overexpresses one or more chaperones.

[0068] In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more additional protease activity, and deficient in one or more autolytic factor activity. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more additional protease activity, and overexpresses one or more inactivated proteases. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more additional protease activity, and overexpresses one or more chaperones.

[0069] In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more autolytic factor activity, and overexpresses one or more chaperones. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, overexpress one or more inactivated proteases, and overexpresses one or more chaperones. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more additional proteases, is deficient in one or more autolytic factor activity, and overexpresses one or more inactivated proteases. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more additional protease activity, is deficient in one or more autolytic factor activity, and overexpresses one or more chaperones. In some embodiments, a recombinant gram-negative bacterial host cell is deficient in a first protease activity and a second protease activity, is deficient in one or more additional protease activity, is deficient in one or more autolytic factor activity, overexpress one or more inactivated proteases, and overexpresses one or more chaperones.

[0070] In some embodiments, an additional protease activity is different from the first protease activity. In some embodiments, an additional protease activity is different from the second protease activity. In some embodiments, an additional deficient protease activity is different from the first protease activity and the second protease activity. In some embodiments, an autolytic factor activity is different from the first protease activity. In some embodiments, an autolytic factor activity is different from the second protease activity. In some embodiments, an autolytic factor activity is different from an additional protease activity. In some embodiments, an autolytic factor activity is different from the normal activity of an overexpressed inactivated protease. In some embodiments, an autolytic factor activity is different from the activity of an overexpressed chaperone. In some embodiments, an autolytic factor activity is different from the

first protease activity and the second protease activity. In some embodiments, the normal activity of an overexpressed inactivated protease is different from the first protease activity. In some embodiments, the normal activity of an overexpressed inactivated protease is different from the second protease activity. In some embodiments, the normal activity of an overexpressed inactivated protease is different from the first protease activity and the second protease activity. In some embodiments, the normal activity of an overexpressed inactivated protease is the same as the first protease activity. In some embodiments, the normal activity of an overexpressed inactivated protease is the same as the second protease activity. In some embodiments, the normal activity of an overexpressed inactivated protease is the same as an additional deficient protease activity.

[0071] In some embodiments, the activity of an overexpressed chaperone is different from the first protease activity. In some embodiments, the activity of an overexpressed chaperone is different from the second protease activity. In some embodiments, the activity of an overexpressed chaperone is different from the first protease activity and the second protease activity. In some embodiments, the activity of an overexpressed chaperone is different from an additional protease activity. In some embodiments, the activity of an overexpressed chaperone is different from an overexpressed inactivated protease activity.

[0072] In some embodiments, a deficiency in a host cell protein activity, e.g., a protease activity or autolytic factor activity, results from a mutation of a gene encoding a protein having the activity. In some embodiments, a deficiency in a protein activity results from the mutation of at least two genes encoding proteins having the activity, wherein each of the at least two genes encodes a related protein as described herein. The related proteins may be homologues, share a minimum sequence similarity or identity, or both. In some embodiments, a deficiency in a protein activity results from mutation of any gene corresponding to any related protein or any protein homologue. In some embodiments, a deficiency in a protease activity results from mutation of a gene corresponding to any related protease and/or protease homologue. In some embodiments, a deficiency in an autolytic factor activity results from mutation of a gene corresponding to any related autolytic factor and/or autolytic factor homologue.

[0073] Bacterial Tail-Specific Proteases

[0074] The present invention provides recombinant gram-negative bacterial host cells deficient in a first protease activity, wherein the first protease activity is a tail-specific protease activity. Tail-specific proteases (Prc/Tsp) are described in, e.g., Exspasy enzyme EC 3.4.21.102, incorporated herein by reference. Prc is an ATP-dependent periplasmic protease designated a tail-specific protease (Tsp) because it cleaves protein C-termini in a sequence-dependent manner as described by, e.g., Kerr, C.H., et al., 2014, "Salinity-Dependent Impacts of ProQ, Prc, and Spr

Deficiencies on *Escherichia coli* Cell Structure, J. Bact. 196(6):1286-1296, incorporated by reference herein. Inactivation of the *prc* gene in *E. coli* confers thermosensitive cell growth under low osmolality (e.g., Hara H., et al., 1991, "Cloning, mapping, and characterization of the *Escherichia coli prc* gene, which is involved in C-terminal processing of penicillin-binding protein 3," J. Bact. 173(15):4799-813, incorporated by reference herein). Some gram-negative bacteria have genes encoding two or more Prc-related proteins or homologues, e.g., *P. fluorescens* Prc1 and Prc2. Tail-specific proteases are found in many gram-negative bacteria, including *Escherichia*, *Vibrio*, *Erwinia*, *Salmonella*, *Klebsiella*, *Legionella* and *Pseudomonads*.

[0075] The tail-specific protease can degrade a recombinant protein expressed in a bacterial host cell. Thus, a recombinant host cell that is deficient in tail-specific protease activity can produce a higher quality recombinant protein of interest than a corresponding host cell having a functional tail-specific protease. For example, antibody fragments produced in bacteria deficient in tail-specific protease activity are less degraded. (See, e.g., the Examples herein, and U.S. Pat. No. 9,493,559, "Bacterial host strain expressing recombinant DsbC and having reduced Tsp activity," each incorporated herein by reference in its entirety.) However, as also shown, recombinant bacterial host cells that are deficient in tail-specific protease activity fail to grow to high cell density. Poor cell growth in turn leads to reduced yield of recombinant protein. The present invention overcomes this problem by further modifying a recombinant host cell deficient in tail-specific protease activity, to introduce a second protease deficiency, thereby allowing growth to high cell density. Also provided are ways to increase recombinant protein yield by additional strategic modifications.

[0076] In some embodiments, the first protease activity is a tail-specific protease activity. A host cell deficient in tail-specific protease activity can be achieved by mutation of a gene encoding a tail-specific protease, tail-specific protease related protein, and/or a tail-specific protease homologue. In some embodiments, tail-specific protease deficiency results from mutation of a gene encoding a *Pseudomonad* Prc. In some embodiments, tail-specific protease deficiency results from mutation of a gene encoding Prc1, a Prc1-related protein, or a Prc1 homologue. In some embodiments, Prc1 has the amino acid sequence of SEQ ID NO: 33. In some embodiments, a Prc1-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 33. In some embodiments, tail-specific protease deficiency results from mutation of a gene encoding Prc2, a Prc2-related protein, or a Prc2 homologue. In some embodiments, the Prc2 has the amino acid sequence of SEQ ID NO: 35. In some embodiments, a Prc2-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 35. In some embodiments, tail-specific protease deficiency results from mutation of a gene encoding both Prc1, a Prc1-related protein, or a Prc1 homologue,

and mutation of a gene encoding Prc2, a Prc2-related protein, or a Prc2 homologue. In some embodiments, tail-specific protease deficiency results from mutation of a gene encoding *E. coli* Prc (Tsp). In some embodiments, tail-specific protease deficiency results from mutation of a gene encoding *E. coli* Tsp, a Tsp-related protein, or a Tsp homologue. In some embodiments, a Tsp has the amino acid sequence of *E. coli* Tsp (SEQ ID NO: 71). In some embodiments, a Tsp-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 71. In some embodiments, a Tsp-related protein is a homologue of SEQ ID NO: 71.

[0077] In some embodiments, the tail-specific protease deficiency results from mutation of a gene encoding any one or more of: a Prc1 having the amino acid sequence of SEQ ID NO: 33, a Prc1-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 33, a homologue of Prc1 having the amino acid sequence of SEQ ID NO: 33, a Prc2 having the amino acid sequence of SEQ ID NO: 35, a Prc1-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 35, a homologue of Prc2 having the amino acid sequence of SEQ ID NO: 35, a Tsp having the amino acid sequence of SEQ ID NO: 71, a Tsp-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 71, and a homologue of Tsp having the amino acid sequence of SEQ ID NO: 71.

[0078] Bacterial Peptidoglycan Hydrolases

[0079] Bacterial peptidoglycan hydrolases cleave bonds in the peptidoglycan sacculus and/or its fragments. Peptidoglycan hydrolase activity is important in the regulation of cell wall growth, the turnover of peptidoglycan during growth, and the separation of daughter cells during cell division and autolysis. Peptidoglycan hydrolases also are involved in lysis phenomena occurring in bacterial populations.

[0080] The peptidoglycan sacculus is composed of glycan strands cross-linked by short peptides, and forms a closed, bag-shaped structure surrounding the cytoplasmic membrane of most bacteria. The glycan strands of the sacculus are made up of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by β 1-4 bonds. The D-lactoyl group of each MurNAc residue is substituted by a peptide stem typically having composition L-Ala- γ -D-Glu-*meso*-A2pm (or L-Lys)-D-Ala-D-Ala (A2pm, 2,6-diaminopimelic acid) in nascent peptidoglycan, the last D-Ala residue absent in the mature macromolecule. See, e.g., Vollmer, W. et al., 2008, "Peptidoglycan structure and architecture," FEMS Micro. Rev. 32:149-167, incorporated herein by reference in its entirety.

[0081] Murein DD-endopeptidases

[0082] In some embodiments, the recombinant gram-negative bacterial host cell of the present invention is deficient in a first protease activity and a second protease activity, wherein the second protease activity is a murein DD-endopeptidase activity. Murein DD-endopeptidases cleave DD-bonds in the stem peptides of the sacculus glycan strands. See, e.g., Vollmer, W. et al., 2008, "Bacterial peptidoglycan (murein) hydrolases," FEMS Micro. Rev. 32:259-286, incorporated herein by reference in its entirety. Murein DD-endopeptidases from many bacteria, including *Escherichia*, *Vibrio*, *Erwinia*, *Salmonella*, *Klebsiella*, *Legionella* and *Pseudomonads*, have been described in the literature.

[0083] A host cell deficient in Murein DD-endopeptidase activity can be achieved by mutation of one or more gene encoding a murein DD-endopeptidase, murein DD-endopeptidase related protein, and/or a murein DD-endopeptidase homologue. In some embodiments, the murein DD-endopeptidase gene encodes a protease having the amino acid sequence of any one of *P. fluorescens* MepM1 (SEQ ID NO: 1), *E. coli* MepM (also referred to as YebA) (SEQ ID NO: 63), *P. aeruginosa* MepM1 (SEQ ID NO: 66), and *P. putida* MepM1 (SEQ ID NO: 65). In some embodiments, murein DD-endopeptidase deficiency results from mutation of a gene encoding a protease having the amino acid sequence of *P. fluorescens* MepM1 (SEQ ID NO: 1), a *P. fluorescens* MepM1-related protein, or a *P. fluorescens* MepM1 homologue. In some embodiments, a murein DD-endopeptidase-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 1. In some embodiments, murein DD-endopeptidase deficiency results from mutation of a gene encoding a protease having the amino acid sequence of *E. coli* MepM (also referred to as YebA) (SEQ ID NO: 63), an *E. coli* MepM-related protein, or an *E. coli* MepM homologue. In some embodiments, murein DD-endopeptidase has the amino acid sequence of SEQ ID NO: 63. In some embodiments, murein DD-endopeptidase-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 63. In some embodiments, murein DD-endopeptidase deficiency results from mutation of a gene encoding a protease having the amino acid sequence of *P. aeruginosa* MepM1 (SEQ ID NO: 66), a *P. aeruginosa* MepM1-related protein, or a *P. aeruginosa* MepM1 homologue. In some embodiments, a murein DD-endopeptidase-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 66. In some embodiments, murein DD-endopeptidase deficiency results from mutation of a gene encoding a protease having the amino acid sequence of *P. putida* MepM1 (SEQ ID NO: 65), a *P. putida* MepM1-related protein, or a *P. putida* MepM1 homologue. In some embodiments, a murein DD-endopeptidase-related protein has at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 65.

[0084] In some embodiments, murein DD-endorpeptidase deficiency results from mutation of a gene encoding any one or more of: a murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 1, a murein DD-endorpeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 1, a homologue of murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 1, a murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 63, a murein DD-endorpeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 63, a homologue of murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 63, a murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 65, a murein DD-endorpeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 65, a homologue of murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 65, a murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 66, a murein DD-endorpeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 66, and a homologue of a murein DD-endorpeptidase having the amino acid sequence of SEQ ID NO: 66.

[0085] The murein DD-endorpeptidase deficiency can result from any one or more mutation in a host cell gene encoding the murein DD-endorpeptidase, as described elsewhere herein, e.g. (i) a complete gene deletion, (ii) a partial gene deletion, (iii) a missense mutation, (iv) a nonsense mutation, (v) a frameshift mutation, (vi) an insertion, or (vii) any combination of (ii), (iii), (iv), (v) and (vi). In some embodiments, the protease deficiency results from a mutation that changes an amino acid in a conserved region of the murein DD-endorpeptidase having the amino acid sequence set forth as SEQ ID NO: 1 or the analogous conserved region of a murein DD-endorpeptidase having at least 60% similarity to the murein DD-endorpeptidase amino acid sequence set forth as SEQ ID NO: 1. **Fig. 10** shows an exemplary amino acid alignment indicating residues conserved across murein DD-endorpeptidases SEQ ID NO: 1 (*P. fluorescens* MepM1), SEQ ID NO: 63 (*E. coli* MepM, also referred to as YebA), SEQ ID NO: 66 (*P. aeruginosa* MepM1), and/or SEQ ID NO: 65 (*P. putida* MepM1). In some embodiments, the deficiency in a murein DD-endorpeptidase results from a mutation that changes or otherwise disrupts (e.g., by substitution, deletion, insertion, or truncation) an amino acid at a conserved position. A conserved position can be identified by one of skill in the art by any known method. In some embodiments, a conserved position is identified by comparison of the murein DD-endorpeptidase amino acid sequence with any one or more of SEQ ID NOS: 1, 63, 66, and 65. For example, the amino acid sequences may be compared as shown in **Fig. 10**, by CLUSTAL Omega using Genious Prime Software. **Fig. 10** shows the aligned corresponding amino acid positions among the compared murein DD-endorpeptidases, including the corresponding

conserved amino acid positions as indicated in white lettering with dark shading. In some embodiments, the mutation is a non-conservative amino acid substitution. As described herein, an amino acid substitution can be a conservative or non-conservative substitution. Conservative and non-conservative amino acid substitutions are described in the literature and can readily be identified by methods well-known to those of skill in the art and as described herein (see, e.g., **Table 2**, listing conservative amino acid substitutions). In some embodiments, a mutation, e.g., a non-conservative amino acid substitution, replaces or otherwise disrupts an amino acid residue in a murein DD-endopeptidase amino acid sequence at a position corresponding to any of the following positions: SEQ ID NO: 1 positions listed in **Table 1**, column 2; SEQ ID NO: 63 positions listed in **Table 1**, column 4; SEQ ID NO: 65 positions listed in **Table 1**, column 8; and SEQ ID NO: 66 positions listed in **Table 1**, column 6. In some embodiments, a mutation, e.g., a non-conservative amino acid substitution, replaces or otherwise disrupts an amino acid residue in a murein DD-endopeptidase amino acid sequence, wherein the replaced or disrupted amino acid residue is an amino acid residue selected from any of: SEQ ID NO: 1 residues listed in **Table 1**, column 3; SEQ ID NO: 63 residues listed in **Table 1**, column 5; SEQ ID NO: 65 residues listed in **Table 1**, column 9; and SEQ ID NO: 66 residues listed in **Table 1**, column 7. Bold text indicates active site conserved positions in each of SEQ ID NOS: 1, 63, 66, and 65.

[0086] Table 1. Fig. 10 Corresponding Conserved Amino Acid Positions

Row	Position	AA Residue	Position	AA Residue	Position	AA Residue	Position	AA Residue
	SEQ ID NO: 1 (<i>P. f.</i>)	SEQ ID NO: 1	SEQ ID NO: 63 (<i>E. coli</i>)	SEQ ID NO: 63	SEQ ID NO: 66 (<i>P. a.</i>)	SEQ ID NO: 66	SEQ ID NO: 65 (<i>P. p.</i>)	SEQ ID NO: 65
1	44	T	46	T	12	T	44	T
2	50	L	54	L	18	L	50	L
3	51	E	55	E	19	E	51	E
4	109	V	100	V	87	V	111	V
5	112	G	103	G	90	G	114	G
6	113	D	104	D	91	D	115	D
7	114	T	105	T	92	T	116	T
8	115	L	106	L	93	L	117	L
9	116	S	107	S	94	S	118	S
10	123	G	114	G	101	G	125	G
11	140	K	128	K	118	K	142	K
12	145	L	133	L	123	L	147	L

13	146	K	134	K	124	K	148	K
14	148	G	136	G	126	G	150	G
15	149	Q	137	Q	127	Q	151	Q
16	155	L	143	L	133	L	157	L
17	159	G	147	G	137	G	161	G
18	161	L	149	L	139	L	163	L
19	164	L	152	L	142	L	166	L
20	169	S	157	S	147	S	171	S
21	172	E	160	E	150	E	174	E
22	182	G	171	G	160	G	184	G
23	201	G	190	G	179	G	203	G
24	206	S	195	S	184	S	208	S
25	214	A	203	A	192	A	216	A
26	215	G	204	G	193	G	217	G
27	216	L	205	L	194	L	218	L
28	233	D	222	D	211	D	235	D
29	234	F	223	F	212	F	236	F
30	241	G	229	G	219	G	243	G
31	242	D	230	D	220	D	244	D
32	243	E	231	E	221	E	245	E
33	244	F	232	F	222	F	246	F
34	246	V	234	V	224	V	248	V
35	255	G	243	G	233	G	257	G
36	256	K	244	K	234	K	258	K
37	264	L	251	L	242	L	266	L
38	267	R	254	R	245	R	269	R
39	272	G	259	G	250	G	274	G
40	273	K	260	K	251	K	275	K
41	277	A	264	A	255	A	279	A
42	279	R	266	R	257	R	281	R
43	290	Y	273	Y	268	Y	292	Y
44	294	G	277	G	272	G	296	G
45	299	K	282	K	277	K	301	K

46	301	F	284	F	279	F	303	F
47	303	R	286	R	281	R	305	R
48	305	P	288	P	283	P	307	P
49	310	R	294	R	288	R	312	R
50	311	I	295	I	289	I	313	I
51	312	S	296	S	290	S	314	S
52	313	S	297	S	291	S	315	S
53	315	F	299	F	293	F	317	F
54	319	R	303	R	297	R	321	R
55	322	P	306	P	300	P	324	P
56	330	H	314	H	308	H	332	H
57	332	G	316	G	310	G	334	G
58	333	V	317	V	311	V	335	V
59	334	D	318	D	312	D	336	D
60	336	A	320	A	314	A	338	A
61	338	P	322	P	316	P	340	P
62	340	G	324	G	318	G	342	G
63	341	T	325	T	319	T	343	T
64	342	P	326	P	320	P	344	P
65	347	G	331	G	325	G	349	G
66	348	D	332	D	326	D	350	D
67	349	G	333	G	327	G	351	G
68	354	A	338	A	332	A	356	A
69	356	R	340	R	334	R	358	R
70	358	G	342	G	336	G	360	G
71	361	G	345	G	339	G	363	G
72	364	V	348	V	342	V	366	V
73	366	I	350	I	344	I	368	I
74	368	H	352	H	346	H	370	H
75	369	G	353	G	347	G	371	G
76	372	Y	356	Y	350	Y	374	Y
77	374	T	358	T	352	T	376	T
78	376	Y	360	Y	354	Y	378	Y

79	378	H	362	H	356	H	380	H
80	389	G	371	G	367	G	391	G
81	392	V	374	V	370	V	394	V
82	393	K	375	K	371	K	395	K
83	395	G	377	G	373	G	397	G
84	398	I	380	I	376	I	400	I
85	402	G	384	G	380	G	404	G
86	404	T	386	T	382	T	406	T
87	405	G	387	G	383	G	407	G
88	408	T	390	T	386	T	410	T
89	409	G	391	G	387	G	411	G
90	410	P	392	P	388	P	412	P
91	411	H	393	H	389	H	413	H
92	412	L	394	L	390	L	414	L
93	413	H	395	H	391	H	415	H
94	414	Y	396	Y	392	Y	416	Y
95	415	E	397	E	393	E	417	E
96	419	N	401	N	397	N	421	N
97	423	V	405	V	401	V	425	V
98	425	P	407	P	403	P	427	P
99	426	L	408	L	404	L	428	L
100	429	K	411	K	407	K	431	K
101	430	L	412	L	408	L	432	L
102	431	P	413	P	409	P	433	P
103	441	R	423	R	419	R	443	R
104	444	F	426	F	422	F	446	F
105	447	Q	429	Q	425	Q	449	Q

[0087] In *Vibrio Cholera*, activity of the major endopeptidase ShyA (homologue of *E. coli* MepM) was reported to be regulated by mutations affecting accessibility of the catalytic site (Shin, J-H et al., “Structural basis of peptidoglycan endopeptidase regulation,” PNAS 117(21): 11692-11702, 2020, incorporated herein by reference, esp. with regard to ShyA active site and allosteric site amino acids and positions). Shin, et al. reported that ShyA forms an open conformation potentially allowing exposure of the active site for substrate binding, and a closed conformation. Based on structural predictions, Shin, et al. postulated that hydrophobic and

electrostatic interactions between ShyA binding Domains 1 and 3 (separated by a linker Domain 2) bring them into close proximity to form the closed conformation. They found that allosteric site mutations, including Domain 2 mutations, that stabilized the inactive conformation resulted in lower ShyA activity. *P. fluorescens* MepM1 has 65% similarity to *Vibrio Cholera* ShyA (SEQ ID NO: 58).

[0088] In some embodiments, the mutation in a gene encoding a murine DD-endopeptidase, homologue thereof, or murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1, 63, 66, or 65, changes or otherwise disrupts an allosteric site amino acid.

[0089] In some embodiments, the mutation in a gene encoding a murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1, 63, 66, or 65 changes or otherwise disrupts an amino acid at an active site position. In some embodiments, the active site position corresponds to any position in the regions 319 to 411 of SEQ ID NO: 1.

[0090] In some embodiments, the mutation in a gene encoding a murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1, 63, 66, or 65 changes or otherwise disrupts an amino acid at a conserved catalytic (active) site position. In some embodiments, the disrupted conserved active site position corresponds to any conserved position in the region 319 to 411 (listed in **Table 1** at rows 54-91). In some embodiments, the active site amino acid residue corresponds to any one of catalytic amino acid residues R319, H330, D334, H378, and H411, of SEQ ID NO: 1. In some embodiments, the mutation results in deletion of an amino acid residue corresponding to catalytic site amino acid residue R319, H330, D334, H378, and H411, and any combination thereof, of SEQ ID NO: 1. In some embodiments, the mutation is a nonsense mutation at a position corresponding to Y248 of SEQ ID NO: 1. In some embodiments, the mutation is a G to S substitution at a position corresponding to 332 of SEQ ID NO: 1. In some embodiments, the mutation is a D to N substitution at a position corresponding to 334 of SEQ ID NO: 1. In some embodiments, the mutation is an A to T substitution at a position corresponding to 337 of SEQ ID NO: 1. In some embodiments, the mutation is an H to Y substitution at a position corresponding to 411 of SEQ ID NO: 1. In some embodiments, the mutation is a P to L substitution at a position corresponding to 410 of SEQ ID NO: 1.

[0091] In some embodiments, the mutation in a gene encoding a murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1, 63, 66, 65, and/or 71 changes or otherwise disrupts an amino acid at an allosteric

site position. In some embodiments, the allosteric site position corresponds to any position in the regions 134 to 145, and 361 to 378 of SEQ ID NO: 1.

[0092] In some embodiments, the mutation in a gene encoding a murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1, 63, 66, 65, and/or 71 changes or otherwise disrupts (e.g., by substitution, deletion, insertion, or truncation) an amino acid at a conserved allosteric site position. In some embodiments, the disrupted conserved allosteric site position corresponds to any conserved position in the regions 134 to 145 (listed in **Table 1** at rows 11 and 12), and 361 to 378 of SEQ ID NO: 1 (listed in **Table 1** at rows 71-79).

[0093] In some embodiments, the mutation in a gene encoding a murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1 changes or otherwise disrupts (e.g., by substitution, deletion, insertion, or truncation) an amino acid corresponding to any position in the regions 134 to 145, 361 to 378, and 319 to 411 of SEQ ID NO: 1. In some embodiments, the mutation results in a non-conservative substitution of an amino acid corresponding to the amino acid in any position in the regions 134 to 145, 361 to 378, and 319 to 411 of SEQ ID NO: 1.

[0094] In some embodiments, the mutation in a gene encoding murein DD-endopeptidase having at least 60% similarity to the murein DD-endopeptidase amino acid sequence set forth as SEQ ID NO: 1 changes or otherwise disrupts (e.g., by substitution, deletion, insertion, or truncation) an amino acid corresponding to any one or more of: K140, L145, Y248, R319, P322, H330, G332, V333, D334, A336, A337, P338, G340, T341, P342, G347, D348, G349, A354, R356, G358, G361, V364, I366, H368, G369, Y372, T374, Y376, H378, G389, V392, K393, G395, I398, G402, T404, G405, T408, G409, P410, and H411, of SEQ ID NO: 1. In some embodiments, the mutation results in a non-conservative substitution of an amino acid corresponding to any one or more of: K140, L145, Y248, R319, P322, H330, G332, V333, D334, A336, A337, P338, G340, T341, P342, G347, D348, G349, A354, R356, G358, G361, V364, I366, H368, G369, Y372, T374, Y376, H378, G389, V392, K393, G395, I398, G402, T404, G405, T408, G409, P410, and H411, of SEQ ID NO: 1.

[0095] MepS/Spr

[0096] An important gram-negative bacterial murein DD-endopeptidase is MepS, also known as Spr in *E. coli*. (See, e.g., ExPASy enzyme EC 3.4.17.13, and Singh, S. K. et al., 2012, “Three redundant murein endopeptidases catalyse an essential cleavage step in peptidoglycan synthesis of *Escherichia coli* K12,” Mol. Microbiol. 86(5): 1036-1051, both incorporated herein by reference.) Truong, T.T. et al., 2020 (“Cell division is antagonized by the activity of peptidoglycan endopeptidases that promote cell elongation,” Mol. Microbiol. 114: 966– 978,

incorporated herein by reference), reported that unchecked MepS protein turnover by tail-specific protease degradation (e.g., in the absence of Prc/Tsp) results in cell growth inhibition and the occurrence of morphological defects during cell division.

[0097] In the context of *E. coli* it was reported that deletion of *prc* inhibits growth at high density fermentation, and that inactivation of MepS by amino acid substitution is required to restore growth to optical densities (ODs) ≥ 200 at 575nm (Hara, H. et al. 1996, U.S. Pat. No. 9,493,559, and EP1341899B1, each incorporated herein by reference). Similarly, complete inactivation of both Prc genes in *Pseudomonads* prevents high cell density growth in bioreactors and cultures fail to grow past OD₅₇₅'s of 20-50 when compared to strains that express both Prc genes, which reach OD₅₇₅ up to 180 in 2L bioreactors (see Examples herein). However, as shown in the Examples, in *P. fluorescens* complete removal of MepS homologues does not rescue the growth defect resulting from the deficiency in Prc activity. Rather, deficiency in MepM1 activity was shown to restore high density cell growth. Furthermore, in *Pseudomonad* host cells deficient in Prc and MepM1 activity, a deficiency in MepS1 had an adverse effect on cell growth, negating the restoration of growth observed upon introduction of the deficiency of MepM1 activity.

[0098] In some embodiments, the present invention provides a recombinant gram-negative bacterial host cell that is: deficient in a first protease activity and a second protease activity, and optionally further (a) is deficient in one or more additional protease activity; (b) is deficient in one or more autolytic factor activity; (c) overexpresses one or more inactivated protease; (d) overexpresses one or more chaperone protein; or (e) any combination of (a), (b), (c) and (d); wherein the host cell produces a functional MepS murein DD-endorpeptidase. In some embodiments, the present invention provides a recombinant gram-negative bacterial host cell that is: deficient in a first protease activity and a second protease activity, and optionally further (a) is deficient in one or more additional protease activity; (b) is deficient in one or more autolytic factor activity; (c) overexpresses one or more inactivated protease; (d) overexpresses one or more chaperone protein; or (e) any combination of (a), (b), (c) and (d); wherein the host cell is not deficient in a MepS murein DD-endorpeptidase. In some embodiments, the recombinant host cell deficient in a first protease activity, a second protease activity, and having a functional MepS murein DD-endorpeptidase and/or not deficient in a MepS murein DD-endorpeptidase is a *Pseudomonad*. In some embodiments, the recombinant host cell deficient in a first protease activity, a second protease activity, having a functional MepS murein DD-endorpeptidase and/or not deficient in a MepS murein DD-endorpeptidase is not *E. coli*. In some embodiments, the functional and/or not deficient MepS murein DD-endorpeptidase has an amino acid sequence set forth as SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 72, or SEQ ID NO: 73, an amino acid

sequence at least 60% similar or at least 60% identical to SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 72, or SEQ ID NO: 73, or is a homologue of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 72, or SEQ ID NO: 73. In some embodiments, the host cell is a *Pseudomonad*, e.g., *P. fluorescens*, and the functional and/or not deficient MepS murein DD-endopeptidase is MepS1 (SEQ ID NO: 5). In some embodiments, the host cell is a *Pseudomonad*, e.g., *P. fluorescens*, and the functional and/or not deficient MepS murein DD-endopeptidase is MepS2 (SEQ ID NO: 7). In some embodiments, the recombinant host cell is a *Pseudomonad*, e.g., *P. fluorescens*, having a functional and/or not deficient MepS1 (SEQ ID NO: 5) and MepS2 (SEQ ID NO: 7).

[0099] Additional Deficient Protein Activities

[0100] As set forth herein, a recombinant gram-negative bacterial host cell deficient in a tail-specific protease activity and a murein-DD-endopeptidase activity can be further: deficient in at least one additional protein activity. Deficiencies in one or more additional protein activities were found to provide higher quality and yield of recombinant protein. These additional proteins include additional proteases, and autolytic factors. In some embodiments, the recombinant gram-negative host cell is deficient in one or more additional protein activity. In some embodiments, the additional protein activity is a protease activity or autolytic factor activity.

[0101] In some embodiments, the recombinant gram-negative host cell deficient in a tail-specific protease activity and a murein-DD-endopeptidase activity is further deficient in 1 to 10 different additional protease activities. In some embodiments, a deficient additional protease activity results from a mutation in at least one gene encoding an additional protease, that has the additional protease activity. In some embodiments, a deficient additional protease activity results from a mutation in at least two genes encoding an additional protease(s) that have the additional protease activity. In some embodiments, the 1-10 different additional protease activities result from mutations in 1 to 30 genes encoding corresponding additional protease(s).

[0102] In some embodiments, the additional protease is a serralyisin precursor (e.g., an extracellular alkaline metalloprotease, e.g., RXF04495.2, or an autolytic serralyisin precursor, e.g., RXF4500), membrane-localized protease (e.g., HtpX, FtsH, OmpT), murein L,D transpeptidase, hemolysin precursor, D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor, periplasmic serine endoprotease (e.g., DegP or DegP2), AAA+ family proteolytic machine (e.g., HslU/HslV), or a murein DD-endopeptidase (e.g., a MepM, e.g., a *Pseudomonad* MepM2).

[0103] In some embodiments, an additional protease is selected from:

[0104] a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO:

9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9;

[0105] a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 47; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 47; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 47;

[0106] a membrane-localized protease that is an HtpX having the amino acid sequence set forth as SEQ ID NO: 39; a homologue of the HtpX having the amino acid sequence set forth as SEQ ID NO: 39; or an HtpX related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 39;

[0107] a murein L,D transpeptidase having the amino acid sequence set forth as SEQ ID NO: 41; a homologue of the murein L,D transpeptidase having the amino acid sequence set forth as SEQ ID NO: 41; or a murein L,D transpeptidase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 41;

[0108] a hemolysin precursor having the amino acid sequence set forth as SEQ ID NO: 43; a homologue of the hemolysin precursor having the amino acid sequence set forth as SEQ ID NO: 43; or a hemolysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 43;

[0109] a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45; a homologue of the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45; or a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 45;

[0110] a periplasmic serine endoprotease that is a DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a homologue of the DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; or a DegP2 related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 31;

[0111] a periplasmic serine endoprotease that is a DegP having the amino acid sequence set forth as SEQ ID NO: 69; a homologue of the DegP2 having the amino acid sequence set forth as SEQ ID NO: 69; or a DegP2 related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 69;

[0112] a periplasmic serine endoprotease that is a DegP having the amino acid sequence set forth as SEQ ID NO: 62; a homologue of the DegP2 having the amino acid sequence set forth as SEQ

ID NO: 62; or a DegP2 related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 62;

[0113] an AAA+ family proteolytic machine that comprises: an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37; a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37; or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37, and an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38; a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38; or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38;

[0114] a murein DD-endopeptidase that is a protease having the amino acid sequence set forth as SEQ ID NO: 3 (*P. fluorescens* MepM2), a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 3, or a MepM2-related protein having at least 60% similarity or 60% sequence identity to the amino acid sequence set forth as SEQ ID NO: 3;

[0115] a murein DD-endopeptidase that is a protease having the amino acid sequence set forth as SEQ ID NO: 64 (*E. coli* MepM2), a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64, or a MepM2-related protein having at least 60% similarity or 60% sequence identity to the amino acid sequence set forth as SEQ ID NO: 64;

[0116] a murein DD-endopeptidase that is a protease having the amino acid sequence set forth as SEQ ID NO: 67 (*P. putida* MepM2), a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67, or a MepM2-related protein having at least 60% similarity or 60% sequence identity to the amino acid sequence set forth as SEQ ID NO: 67; and

[0117] a murein DD-endopeptidase that is a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68 (*P. aeruginosa*), a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68, or a MepM2-related protein having at least 60% similarity or 60% sequence identity to the amino acid sequence set forth as SEQ ID NO: 68.

[0118] A deficiency in an additional protease activity may result from a mutation in an aminopeptidase; dipeptidase; dipeptidyl-peptidase; tripeptidyl peptidase; peptidyl-dipeptidase; serine-type carboxypeptidase; metallo carboxypeptidase; cysteine-type carboxypeptidase; omegapeptidase; serine proteinase; cysteine proteinase; aspartic proteinase; metallo proteinase; or a proteinase of unknown mechanism.

[0119] Aminopeptidases include cytosol aminopeptidase (leucyl aminopeptidase), membrane alanyl aminopeptidase, cystinyl aminopeptidase, tripeptide aminopeptidase, prolyl aminopeptidase, arginyl aminopeptidase, glutamyl aminopeptidase, x-pro aminopeptidase, bacterial leucyl aminopeptidase, thermophilic aminopeptidase, clostridial aminopeptidase,

cytosol alanyl aminopeptidase, lysyl aminopeptidase, x-trp aminopeptidase, tryptophanyl aminopeptidase, methionyl aminopeptidase, d-stereospecific aminopeptidase, aminopeptidase ey. Dipeptidases include x-his dipeptidase, x-arg dipeptidase, x-methyl-his dipeptidase, cys-gly dipeptidase, glu-glu dipeptidase, pro-x dipeptidase, x-pro dipeptidase, met-x dipeptidase, non-stereospecific dipeptidase, cytosol non-specific dipeptidase, membrane dipeptidase, beta-ala-his dipeptidase. Dipeptidyl-peptidases and tripeptidyl peptidases include dipeptidyl-peptidase i, dipeptidyl-peptidase ii, dipeptidyl peptidase iii, dipeptidyl-peptidase iv, dipeptidyl-dipeptidase, tripeptidyl-peptidase I, tripeptidyl-peptidase II. Peptidyl-dipeptidases include peptidyl-dipeptidase a and peptidyl-dipeptidase b. Serine-type carboxypeptidases include lysosomal pro-x carboxypeptidase, serine-type D-ala-D-ala carboxypeptidase, carboxypeptidase C, carboxypeptidase D. Metallo-carboxypeptidases include carboxypeptidase a, carboxypeptidase B, lysine(arginine) carboxypeptidase, gly-X carboxypeptidase, alanine carboxypeptidase, muramoylpentapeptide carboxypeptidase, carboxypeptidase h, glutamate carboxypeptidase, carboxypeptidase M, muramoyltetrapeptide carboxypeptidase, zinc d-ala-d-ala carboxypeptidase, carboxypeptidase A2, membrane pro-x carboxypeptidase, tubuliny-tyr carboxypeptidase, carboxypeptidase t. Omegapeptidases include acylaminoacyl-peptidase, peptidyl-glycinamidase, pyroglutamyl-peptidase I, beta-aspartyl-peptidase, pyroglutamyl-peptidase II, n-formylmethionyl-peptidase, pteroylpoly-[gamma]-glutamate carboxypeptidase, gamma-glu-X carboxypeptidase, acylmuramoyl-ala peptidase. Serine proteinases include chymotrypsin, chymotrypsin c, metridin, trypsin, thrombin, coagulation factor Xa, plasmin, enteropeptidase, acrosin, alpha-lytic protease, glutamyl, endopeptidase, cathepsin G, coagulation factor viia, coagulation factor ixa, cucumisi, prolyl oligopeptidase, coagulation factor xia, bradykinin, plasma kallikrein, tissue kallikrein, pancreatic elastase, leukocyte elastase, coagulation factor xiaa, chymase, complement component c1r55, complement component c1s55, classical-complement pathway c3/c5 convertase, complement factor I, complement factor D, alternative-complement pathway c3/c5 convertase, cerevisin, hypodermin C, lysyl endopeptidase, endopeptidase 1a, gamma-renin, venombin ab, leucyl endopeptidase, tryptase, scutellarin, kexin, subtilisin, oryzin, endopeptidase k, thermomycolin, thermitase, endopeptidase SO, T-plasminogen activator, protein C, pancreatic endopeptidase E, pancreatic elastase ii, IGA-specific serine endopeptidase, U-plasminogen, activator, venombin A, furin, myeloblastin, semenogelase, granzyme A or cytotoxic T-lymphocyte proteinase 1, granzyme B or cytotoxic T-lymphocyte proteinase 2, streptogrisin A, streptogrisin B, glutamyl endopeptidase II, oligopeptidase B, limulus clotting factor c, limulus clotting factor, limulus clotting enzyme, omptin, repressor lexa, bacterial leader peptidase I, togavirin, flavirin. Cysteine proteinases include cathepsin B, papain, ficin, chymopapain, asclepain, clostripain, streptopain, actinide, cathepsin 1, cathepsin H,

calpain, cathepsin t, glycyl, endopeptidase, cancer procoagulant, cathepsin S, picornain 3C, picornain 2A, caricain, ananain, stem bromelain, fruit bromelain, legumain, histolysin, interleukin 1-beta converting enzyme. Aspartic proteinases include pepsin A, pepsin B, gastricsin, chymosin, cathepsin D, neopenthesin, renin, retropepsin, pro-opiomelanocortin converting enzyme, aspergillopepsin I, aspergillopepsin II, penicillopepsin, rhizopuspepsin, endothiapepsin, mucoropepsin, candidapepsin, saccharopepsin, rhodotorulapepsin, physaropepsin, acrocylindropepsin, polyporopepsin, pycnoporopepsin, scytalidopepsin a, scytalidopepsin b, xanthomonapepsin, cathepsin e, barrierpepsin, bacterial leader peptidase I, pseudomonapepsin, plasmepsin. Metallo proteinases include atrolysin a, microbial collagenase, leucolysin, interstitial collagenase, neprilysin, envelysin, iga-specific metalloendopeptidase, procollagen N-endopeptidase, thimet oligopeptidase, neurolysin, stromelysin 1, meprin A, procollagen C-endopeptidase, peptidyl-lys metalloendopeptidase, astacin, stromelysin, 2, matrilysin gelatinase, aeromonolysin, pseudolysin, thermolysin, bacillolysin, aureolysin, coccolysin, mycolysin, beta-lytic metalloendopeptidase, peptidyl-asp metalloendopeptidase, neutrophil collagenase, gelatinase B, leishmanolysin, saccharolysin, autolysin, deuterolysin, serralysin, atrolysin B, atrolysin C, atroxase, atrolysin E, atrolysin F, adamalysin, horrilysin, ruberlysin, bothropasin, bothrolysin, ophiolysin, trimerelysin I, trimerelysin II, mucrolysin, pitrilysin, insulysin, O-sialoglycoprotein endopeptidase, russellysin, mitochondrial, intermediate, peptidase, dactylisin, nardilysin, magnolysin, meprin B, mitochondrial processing peptidase, macrophage elastase, choriolysin, toxilysin. Proteinases of unknown mechanism include thermopain and multicatalytic endopeptidase complex.

[0120] Certain proteases have both protease and chaperone-like activity. When these proteases are negatively affecting protein yield and/or quality it is often useful to specifically delete their protease activity, and they are overexpressed when their chaperone activity may positively affect protein yield and/or quality. These proteases include, but are not limited to: Hsp100(Clp/Hsl) family members RXF04587.1 (clpA), RXF08347.1, RXF04654.2 (clpX), RXF04663.1, RXF01957.2 (hslU), RXF01961.2 (hslV); Peptidyl-prolyl cis-trans isomerase family member RXF05345.2 (ppiB); Metallopeptidase M20 family member RXF04892.1 (aminohydrolase); Metallopeptidase M24 family members RXF04693.1 (methionine aminopeptidase) and RXF03364.1 (methionine aminopeptidase); and Serine Peptidase S26 signal peptidase I family member RXF01181.1 (signal peptidase).

[0121] These and other proteases and folding modulators are known in the art and described in the literature, e.g., in U.S. Pat. No. 8,603,824, "Process for improved protein expression by strain engineering," incorporated by reference in its entirety. For example, Table D of the '824 patent describes Tig (tig, Trigger factor, FKBP type ppiase (ec 5.2.1.8) RXF04655, UniProtKB -

P0A850 (TIG_ECOLI)). U.S. Pat. Nos. 9,394,571 and 9,580,719, both titled “Method for Rapidly Screening Microbial Hosts to Identify Certain Strains with Improved Yield and/or Quality in the Expression of Heterologous Proteins,” describe Tig (RXF04655.2, SEQ ID NO: 34 therein), LepB (RXF01181.1, SEQ ID NO: 56 therein), DegP1 (RXF01250, SEQ ID NO: 57 therein), AprA (RXF04304.1, SEQ ID NO: 86 therein), Prc1 (RXF06586.1, SEQ ID NO: 120 therein), DegP2, (RXF07210.1, SEQ ID NO: 124 therein), Lon (RXF04653, SEQ ID NO: 92 therein); DsbA (RXF01002.1, SEQ ID NO: 25 therein), and DsbC (RXF03307.1, SEQ ID NO: 26 therein). These sequences and those for other proteases and folding modulators also are set forth in U.S. Pat. No. 9,580,719 (Table of SEQ ID NOS in columns 93-98 therein), incorporated herein by reference in its entirety. For example, U.S. Pat. No. 9,580,719 provides the sequence encoding HslU (RXF01957.2) and HslV (RXF01961.2) as SEQ ID NOS 18 and 19, respectively.

[0122] In some embodiments, the recombinant gram-negative host cell is deficient in 1 additional protease activity to 10 additional protease activities. In some embodiments, the recombinant gram-negative host cell is deficient in 1 additional protease activity to 10 additional protease activities. In some embodiments, the recombinant gram-negative host cell is deficient in 1 additional protease activity to 2 additional protease activities, 1 additional protease activity to 3 additional protease activities, 1 additional protease activity to 4 additional protease activities, 1 additional protease activity to 5 additional protease activities, 1 additional protease activity to 6 additional protease activities, 1 additional protease activity to 7 additional protease activities, 1 additional protease activity to 8 additional protease activities, 1 additional protease activity to 9 additional protease activities, 1 additional protease activity to 10 additional protease activities, 2 additional protease activities to 3 additional protease activities, 2 additional protease activities to 4 additional protease activities, 2 additional protease activities to 5 additional protease activities, 2 additional protease activities to 6 additional protease activities, 2 additional protease activities to 7 additional protease activities, 2 additional protease activities to 8 additional protease activities, 2 additional protease activities to 9 additional protease activities, 2 additional protease activities to 10 additional protease activities, 3 additional protease activities to 4 additional protease activities, 3 additional protease activities to 5 additional protease activities, 3 additional protease activities to 6 additional protease activities, 3 additional protease activities to 7 additional protease activities, 3 additional protease activities to 8 additional protease activities, 3 additional protease activities to 9 additional protease activities, 3 additional protease activities to 10 additional protease activities, 4 additional protease activities to 5 additional protease activities, 4 additional protease activities to 6 additional protease activities, 4 additional protease activities to 7 additional protease activities, 4 additional protease activities to 8 additional protease activities, 4 additional protease activities to 9 additional protease activities, 4 additional protease

activities to 10 additional protease activities, 5 additional protease activities to 6 additional protease activities, 5 additional protease activities to 7 additional protease activities, 5 additional protease activities to 8 additional protease activities, 5 additional protease activities to 9 additional protease activities, 5 additional protease activities to 10 additional protease activities, 6 additional protease activities to 7 additional protease activities, 6 additional protease activities to 8 additional protease activities, 6 additional protease activities to 9 additional protease activities, 6 additional protease activities to 10 additional protease activities, 7 additional protease activities to 8 additional protease activities, 7 additional protease activities to 9 additional protease activities, 7 additional protease activities to 10 additional protease activities, 8 additional protease activities to 9 additional protease activities, 8 additional protease activities to 10 additional protease activities, or 9 additional protease activities to 10 additional protease activities. In some embodiments, the recombinant gram-negative host cell is deficient in 1 additional protease activity, 2 additional protease activities, 3 additional protease activities, 4 additional protease activities, 5 additional protease activities, 6 additional protease activities, 7 additional protease activities, 8 additional protease activities, 9 additional protease activities, or 10 additional protease activities. In some embodiments, the recombinant gram-negative host cell is deficient in at least 1 additional protease activity, 2 additional protease activities, 3 additional protease activities, 4 additional protease activities, 5 additional protease activities, 6 additional protease activities, 7 additional protease activities, 8 additional protease activities, or 9 additional protease activities. In some embodiments, the recombinant gram-negative host cell is deficient in at most 2 additional protease activities, 3 additional protease activities, 4 additional protease activities, 5 additional protease activities, 6 additional protease activities, 7 additional protease activities, 8 additional protease activities, 9 additional protease activities, or 10 additional protease activities.

[0123] In some embodiments, a deficient additional protease activity results from a mutation in 1 additional protease gene to 30 additional protease genes. In some embodiments, a deficient additional protease activity results from a mutation in 1 additional protease gene to 2 additional protease genes, 1 additional protease gene to 3 additional protease genes, 1 additional protease gene to 4 additional protease genes, 1 additional protease gene to 5 additional protease genes, 1 additional protease gene to 6 additional protease genes, 1 additional protease gene to 8 additional protease genes, 1 additional protease gene to 10 additional protease genes, 1 additional protease gene to 15 additional protease genes, 1 additional protease gene to 20 additional protease genes, 1 additional protease gene to 25 additional protease genes, 1 additional protease gene to 30 additional protease genes, 2 additional protease genes to 3 additional protease genes, 2 additional protease genes to 4 additional protease genes, 2 additional protease genes to 5 additional protease genes, 2 additional protease genes to 6 additional protease genes, 2 additional protease genes to 8

additional protease genes, 2 additional protease genes to 10 additional protease genes, 2 additional protease genes to 15 additional protease genes, 2 additional protease genes to 20 additional protease genes, 2 additional protease genes to 25 additional protease genes, 2 additional protease genes to 30 additional protease genes, 3 additional protease genes to 4 additional protease genes, 3 additional protease genes to 5 additional protease genes, 3 additional protease genes to 6 additional protease genes, 3 additional protease genes to 8 additional protease genes, 3 additional protease genes to 10 additional protease genes, 3 additional protease genes to 15 additional protease genes, 3 additional protease genes to 20 additional protease genes, 3 additional protease genes to 25 additional protease genes, 3 additional protease genes to 30 additional protease genes, 4 additional protease genes to 5 additional protease genes, 4 additional protease genes to 6 additional protease genes, 4 additional protease genes to 8 additional protease genes, 4 additional protease genes to 10 additional protease genes, 4 additional protease genes to 15 additional protease genes, 4 additional protease genes to 20 additional protease genes, 4 additional protease genes to 25 additional protease genes, 4 additional protease genes to 30 additional protease genes, 5 additional protease genes to 6 additional protease genes, 5 additional protease genes to 8 additional protease genes, 5 additional protease genes to 10 additional protease genes, 5 additional protease genes to 15 additional protease genes, 5 additional protease genes to 20 additional protease genes, 5 additional protease genes to 25 additional protease genes, 5 additional protease genes to 30 additional protease genes, 6 additional protease genes to 8 additional protease genes, 6 additional protease genes to 10 additional protease genes, 6 additional protease genes to 15 additional protease genes, 6 additional protease genes to 20 additional protease genes, 6 additional protease genes to 25 additional protease genes, 6 additional protease genes to 30 additional protease genes, 8 additional protease genes to 10 additional protease genes, 8 additional protease genes to 15 additional protease genes, 8 additional protease genes to 20 additional protease genes, 8 additional protease genes to 25 additional protease genes, 8 additional protease genes to 30 additional protease genes, 10 additional protease genes to 15 additional protease genes, 10 additional protease genes to 20 additional protease genes, 10 additional protease genes to 25 additional protease genes, 10 additional protease genes to 30 additional protease genes, 15 additional protease genes to 20 additional protease genes, 15 additional protease genes to 25 additional protease genes, 15 additional protease genes to 30 additional protease genes, 20 additional protease genes to 25 additional protease genes, 20 additional protease genes to 30 additional protease genes, or 25 additional protease genes to 30 additional protease genes. In some embodiments, a deficient additional protease activity results from a mutation in 1 additional protease gene, 2 additional protease genes, 3 additional protease genes, 4 additional protease genes, 5 additional protease

genes, 6 additional protease genes, 8 additional protease genes, 10 additional protease genes, 15 additional protease genes, 20 additional protease genes, 25 additional protease genes, or 30 additional protease genes. In some embodiments, a deficient additional protease activity results from a mutation in at least 1 additional protease gene, 2 additional protease genes, 3 additional protease genes, 4 additional protease genes, 5 additional protease genes, 6 additional protease genes, 8 additional protease genes, 10 additional protease genes, 15 additional protease genes, 20 additional protease genes, or 25 additional protease genes. In some embodiments, a deficient additional protease activity results from a mutation in at most 2 additional protease genes, 3 additional protease genes, 4 additional protease genes, 5 additional protease genes, 6 additional protease genes, 8 additional protease genes, 10 additional protease genes, 15 additional protease genes, 20 additional protease genes, 25 additional protease genes, or 30 additional protease genes.

[0124] In some embodiments, e.g., in *E. coli*, the additional protease activity results from a mutation in a murein DD-endopeptidase gene encoding one or more of: a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 5 (*Pseudomonad* MepS1), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 5, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 5, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 7 (*Pseudomonad* MepS2), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 7, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 7, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 72 (*Pseudomonad* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 72, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 72, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 73 (*Pseudomonad* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 73, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 73, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32 (*E. coli* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 32, or a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32.

[0125] In some embodiments, the recombinant gram-negative host cell deficient in a tail-specific protease activity and a murein-DD-endopeptidase activity is further deficient in 1 to 10 different autolytic factor activities. In some embodiments, a deficient autolytic factor activity results from

a mutation in at least one gene encoding an autolytic factor, that has the corresponding autolytic factor activity. In some embodiments, a deficient autolytic factor activity results from a mutation in at least two genes encoding autolytic factor(s) that have the autolytic factor activity. In some embodiments, the 1-10 different autolytic factor activities result from mutations in 1 to 30 genes encoding corresponding autolytic factor(s).

[0126] In some embodiments, the autolytic factor is an S-type pyocin, linear gramicidin synthase subunit D, hemolysin precursor, leukotoxin, or porin.

[0127] In some embodiments, the autolytic factor is selected from:

[0128] an S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49; a homologue of the S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49; or an S-type Pyocin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 49;

[0129] a linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51; a homologue of the linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51; or a linear gramicidin synthase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 51;

[0130] a leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53; a homologue of the leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53; or a leukotoxin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 53; and

[0131] an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55; a homologue of the an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55; or an ShlB hemolysin transporter related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 55.

[0132] In some embodiments, the recombinant gram-negative host cell is deficient in 1 autolytic factor activity to 10 autolytic factor activities. In some embodiments, the recombinant gram-negative host cell is deficient in 1 autolytic factor activity to 2 autolytic factor activities, 1 autolytic factor activity to 3 autolytic factor activities, 1 autolytic factor activity to 4 autolytic factor activities, 1 autolytic factor activity to 5 autolytic factor activities, 1 autolytic factor activity to 6 autolytic factor activities, 1 autolytic factor activity to 7 autolytic factor activities, 1 autolytic factor activity to 8 autolytic factor activities, 1 autolytic factor activity to 9 autolytic factor activities, 1 autolytic factor activity to 10 autolytic factor activities, 2 autolytic factor activities to 3 autolytic factor activities, 2 autolytic factor activities to 4 autolytic factor activities, 2 autolytic factor activities to 5 autolytic factor activities, 2 autolytic factor activities to 6 autolytic factor activities, 2 autolytic factor activities to 7 autolytic factor activities, 2 autolytic

factor activities to 8 autolytic factor activities, 2 autolytic factor activities to 9 autolytic factor activities, 2 autolytic factor activities to 10 autolytic factor activities, 3 autolytic factor activities to 4 autolytic factor activities, 3 autolytic factor activities to 5 autolytic factor activities, 3 autolytic factor activities to 6 autolytic factor activities, 3 autolytic factor activities to 7 autolytic factor activities, 3 autolytic factor activities to 8 autolytic factor activities, 3 autolytic factor activities to 9 autolytic factor activities, 3 autolytic factor activities to 10 autolytic factor activities, 4 autolytic factor activities to 5 autolytic factor activities, 4 autolytic factor activities to 6 autolytic factor activities, 4 autolytic factor activities to 7 autolytic factor activities, 4 autolytic factor activities to 8 autolytic factor activities, 4 autolytic factor activities to 9 autolytic factor activities, 4 autolytic factor activities to 10 autolytic factor activities, 5 autolytic factor activities to 6 autolytic factor activities, 5 autolytic factor activities to 7 autolytic factor activities, 5 autolytic factor activities to 8 autolytic factor activities, 5 autolytic factor activities to 9 autolytic factor activities, 5 autolytic factor activities to 10 autolytic factor activities, 6 autolytic factor activities to 7 autolytic factor activities, 6 autolytic factor activities to 8 autolytic factor activities, 6 autolytic factor activities to 9 autolytic factor activities, 6 autolytic factor activities to 10 autolytic factor activities, 7 autolytic factor activities to 8 autolytic factor activities, 7 autolytic factor activities to 9 autolytic factor activities, 7 autolytic factor activities to 10 autolytic factor activities, 8 autolytic factor activities to 9 autolytic factor activities, 8 autolytic factor activities to 10 autolytic factor activities, or 9 autolytic factor activities to 10 autolytic factor activities. In some embodiments, the recombinant gram-negative host cell is deficient in 1 autolytic factor activity, 2 autolytic factor activities, 3 autolytic factor activities, 4 autolytic factor activities, 5 autolytic factor activities, 6 autolytic factor activities, 7 autolytic factor activities, 8 autolytic factor activities, 9 autolytic factor activities, or 10 autolytic factor activities. In some embodiments, the recombinant gram-negative host cell is deficient in at least 1 autolytic factor activity, 2 autolytic factor activities, 3 autolytic factor activities, 4 autolytic factor activities, 5 autolytic factor activities, 6 autolytic factor activities, 7 autolytic factor activities, 8 autolytic factor activities, or 9 autolytic factor activities. In some embodiments, the recombinant gram-negative host cell is deficient in at most 2 autolytic factor activities, 3 autolytic factor activities, 4 autolytic factor activities, 5 autolytic factor activities, 6 autolytic factor activities, 7 autolytic factor activities, 8 autolytic factor activities, 9 autolytic factor activities, or 10 autolytic factor activities.

[0133] In some embodiments, a deficient additional protease activity results from a mutation in 1 autolytic factor gene to 30 autolytic factor genes. In some embodiments, a deficient additional protease activity results from a mutation in 1 autolytic factor gene to 2 autolytic factor genes, 1 autolytic factor gene to 3 autolytic factor genes, 1 autolytic factor gene to 4 autolytic factor

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factor genes. In some embodiments, a deficient additional protease activity results from a mutation in 1 autolytic factor gene, 2 autolytic factor genes, 3 autolytic factor genes, 4 autolytic factor genes, 5 autolytic factor genes, 6 autolytic factor genes, 8 autolytic factor genes, 10 autolytic factor genes, 15 autolytic factor genes, 20 autolytic factor genes, 25 autolytic factor genes, or 30 autolytic factor genes. In some embodiments, a deficient additional protease activity results from a mutation in at least 1 autolytic factor gene, 2 autolytic factor genes, 3 autolytic factor genes, 4 autolytic factor genes, 5 autolytic factor genes, 6 autolytic factor genes, 8 autolytic factor genes, 10 autolytic factor genes, 15 autolytic factor genes, 20 autolytic factor genes, or 25 autolytic factor genes. In some embodiments, a deficient additional protease activity results from a mutation in at most 2 autolytic factor genes, 3 autolytic factor genes, 4 autolytic factor genes, 5 autolytic factor genes, 6 autolytic factor genes, 8 autolytic factor genes, 10 autolytic factor genes, 15 autolytic factor genes, 20 autolytic factor genes, 25 autolytic factor genes, or 30 autolytic factor genes.

[0134] In some embodiments, the deficiency in an additional protein activity results from a mutation in a gene encoding an additional protein that is different from the first and/or second protease. In some embodiments, the mutation is not in a gene encoding a tail-specific protease. In some embodiments, the additional protein deficiency does not result from mutation of a gene encoding any one or more of: a Prc1 having the amino acid sequence of SEQ ID NO: 33, a Prc1-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 33, a homologue of Prc1 having the amino acid sequence of SEQ ID NO: 33, a Prc2 having the amino acid sequence of SEQ ID NO: 35, a Prc1-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 35, a homologue of Prc2 having the amino acid sequence of SEQ ID NO: 35, a Tsp having the amino acid sequence of SEQ ID NO: 71, a Tsp-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 71, and a homologue of Tsp having the amino acid sequence of SEQ ID NO: 71. In some embodiments, the mutation is in a gene encoding a protein having an amino acid sequence that has less than 60% similarity or identity, less than 50% similarity or identity, less than 40% similarity or identity, or less than 30% similarity or identity, to the amino acid sequence of any one of SEQ ID NOS: 33, 35, and 71.

[0135] In some embodiments, the deficiency in an additional protein (e.g., protease or autolytic factor) activity results from a mutation in a gene encoding a murein DD-endopeptidase that is different from a gene encoding the first protease and/or the second protease. In some embodiments, the deficiency in an additional protein activity does not result from a mutation in a gene encoding a MepM1 murein DD-endopeptidase. In some embodiments, in any one or more gram-negative bacterial host cell, the mutation is not in a gene encoding any one or more of: a

murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 1 (MepM), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 1, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 1, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 63 (MepM), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 63, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 63, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 65 (MepM), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 65, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 65, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 66 (MepM), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 66, a homologue of a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 66, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 5 (MepS1), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 5, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 5, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 7 (MepS2), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 7, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 7, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 72 (*Pseudomonad* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 72, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 72, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 73 (*Pseudomonad* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 73, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 73, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32 (*E. coli* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 32, or a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32. In these embodiments, the mutation may be in a gene encoding a murein DD-endopeptidase having an amino acid sequence that has less than 60% similarity or identity, less than 50% similarity or

identity, less than 40% similarity or identity, or less than 30% similarity or identity, to the amino acid sequence of any one of SEQ ID NOS: 1, 63, 65, 66, 5, 7, 72, 73, and 32.

[0136] In some embodiments, in any one or more gram-negative bacterial host cell, e.g., in *E. coli* or a *Pseudomonad*, the deficiency in an additional protein activity (e.g., protease) results from a mutation in a gene encoding any one or more of: a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 5 (MepS1), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 5, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 5, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 7 (MepS2), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 7, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 7, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 72 (*Pseudomonad* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 72, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 72, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 73 (*Pseudomonad* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 73, a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 73, a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32 (*E. coli* MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 32, and a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32. In some embodiments, the gram-negative bacterial host cell is *E. coli*, and the deficiency in an additional protein activity results from a mutation in a gene encoding a murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32 (MepS), a murein DD-endopeptidase-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence of SEQ ID NO: 32, and a homologue of murein DD-endopeptidase having the amino acid sequence of SEQ ID NO: 32, wherein the mutation is not at an amino acid that is or that corresponds to an amino acid selected from: D133, H145, H157, N31, R62, I70, Q73, C94, S95, V98, Q99, R100, L108, Y115, V135, L136, G140, R144, and G147. In related embodiments, the mutation is not a mutation that is or that corresponds to a mutation resulting in N31Y, R62C, I70T, Q73R, C94A, S95F, V98E, Q99P, R100G, L108S, Y115F, D133A, V135D, V135G, L136P, G140C, R144C, H145A, G147C or H157A.

[0137] Overexpressed Proteins

[0138] A recombinant gram-negative bacterial host cell of the invention may overexpress one or more proteins, e.g., an inactivated protease or a folding modulator, e.g., a chaperone. When co-overexpressed with the recombinant protein of interest in the host cell, the overexpressed protein can improve the quality and/or yield of a recombinant protein of interest produced. In some embodiments, the co-overexpressed protein is expressed from an exogenous expression construct. In some embodiments, the expression construct is in a plasmid or expression vector. In some embodiments, when overexpressed in a host cell that also overexpresses the recombinant protein of interest, the co-overexpressed protein and the recombinant protein of interest are expressed from different plasmids. In some embodiments, the co-overexpressed protein and the recombinant protein of interest are expressed from the same plasmid. In some embodiments, the co-overexpressed protein and the recombinant protein of interest are expressed by transcription from different promoters on the same plasmid. In some embodiments, the co-overexpressed protein and the recombinant protein of interest are co-transcribed, that is, they are expressed by transcription from the same promoter on the same plasmid. In some embodiments, the co-overexpressed protein is not expressed from the bacterial chromosome. In some embodiments, the one or more co-overexpressed protein is an inactivated protease. In some embodiments, the one or more co-overexpressed protein is a chaperone or protein folding modulator. In some embodiments, the recombinant gram-negative host cell overexpresses 1 co-overexpressed protein to 20 different co-overexpressed proteins. In some embodiments, the recombinant gram-negative host cell overexpresses 1 co-overexpressed protein to 2 different co-overexpressed proteins, 1 co-overexpressed protein to 3 different co-overexpressed proteins, 1 co-overexpressed protein to 4 different co-overexpressed proteins, 1 co-overexpressed protein to 5 different co-overexpressed proteins, 1 co-overexpressed protein to 6 different co-overexpressed proteins, 1 co-overexpressed protein to 7 different co-overexpressed proteins, 1 co-overexpressed protein to 8 different co-overexpressed proteins, 1 co-overexpressed protein to 9 different co-overexpressed proteins, 1 co-overexpressed protein to 10 different co-overexpressed proteins, 1 co-overexpressed protein to 15 different co-overexpressed proteins, 1 co-overexpressed protein to 20 different co-overexpressed proteins, 2 different co-overexpressed proteins to 3 different co-overexpressed proteins, 2 different co-overexpressed proteins to 4 different co-overexpressed proteins, 2 different co-overexpressed proteins to 5 different co-overexpressed proteins, 2 different co-overexpressed proteins to 6 different co-overexpressed proteins, 2 different co-overexpressed proteins to 7 different co-overexpressed proteins, 2 different co-overexpressed proteins to 8 different co-overexpressed proteins, 2 different co-overexpressed proteins to 9 different co-overexpressed proteins, 2 different co-overexpressed proteins to 10 different co-overexpressed proteins, 2 different co-overexpressed proteins to 15 different co-overexpressed proteins, 2

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proteins, 9 different co-overexpressed proteins to 15 different co-overexpressed proteins, 9 different co-overexpressed proteins to 20 different co-overexpressed proteins, 10 different co-overexpressed proteins to 15 different co-overexpressed proteins, 10 different co-overexpressed proteins to 20 different co-overexpressed proteins, or 15 different co-overexpressed proteins to 20 different co-overexpressed proteins. In some embodiments, the recombinant gram-negative host cell overexpresses 1 co-overexpressed protein, 2 different co-overexpressed proteins, 3 different co-overexpressed proteins, 4 different co-overexpressed proteins, 5 different co-overexpressed proteins, 6 different co-overexpressed proteins, 7 different co-overexpressed proteins, 8 different co-overexpressed proteins, 9 different co-overexpressed proteins, 10 different co-overexpressed proteins, 15 different co-overexpressed proteins, or 20 different co-overexpressed proteins. In some embodiments, the recombinant gram-negative host cell overexpresses at least 1 co-overexpressed protein, 2 different co-overexpressed proteins, 3 different co-overexpressed proteins, 4 different co-overexpressed proteins, 5 different co-overexpressed proteins, 6 different co-overexpressed proteins, 7 different co-overexpressed proteins, 8 different co-overexpressed proteins, 9 different co-overexpressed proteins, 10 different co-overexpressed proteins, or 15 different co-overexpressed proteins. In some embodiments, the recombinant gram-negative host cell overexpresses at most 2 different co-overexpressed proteins, 3 different co-overexpressed proteins, 4 different co-overexpressed proteins, 5 different co-overexpressed proteins, 6 different co-overexpressed proteins, 7 different co-overexpressed proteins, 8 different co-overexpressed proteins, 9 different co-overexpressed proteins, 10 different co-overexpressed proteins, 15 different co-overexpressed proteins, or 20 different co-overexpressed proteins.

[0139] Inactivated Proteases

[0140] In some embodiments, the one or more co-overexpressed protein is an inactivated protease. An inactivated protease derived from a functional protease present in the host cell can be overexpressed by a host cell to reduce the functional protease activity in a host cell. The inactivated protease mutant can act as dominant negative protease. The overexpressed inactivated protease can be exogenously produced, e.g., from an expression construct on a plasmid. In some embodiments, the recombinant gram-negative host cell overexpresses 1 to 10 different inactivated proteases. In some embodiments, an overexpressed inactivated protease is inactivated by a mutation in a gene encoding the corresponding functional protease.

[0141] In some embodiments, an inactivated protease is an inactive form of a gram negative bacterial a serine protease gene from the EC 3.4.21.107 enzyme family. In some embodiments, an inactivated protease is a DegP protease (also known as HtrA). A DegP protease can be, e.g., a DegP2 protease, or a DegP-like protease. DegP proteases are periplasmic serine endoproteases.

Their structure is described, e.g., by Pallen, M.J. and Wren, B.W., 1997, "The HtrA family of serine proteases," *Molecular Microbiology* 26(2): 209–221, both incorporated herein by reference. In some embodiments, the DegP protease is inactivated by mutation in a gene encoding a DegP protease selected from: *P. fluorescens* DegP2 (SEQ ID NO: 31); *P. fluorescens* DegP (SEQ ID NO: 69); *E. coli* DegP/HtrA (SEQ ID NO: 62); or *P. putida* DegP (e.g., UniProtKB – A5W8F5 strain, *P. putida* F1, or B0KV30, strain *P. putida* GB1).

[0142] In some embodiments, an overexpressed inactivated protease is inactivated by a mutation in a gene encoding a DegP, a DegP-related protein, or a DegP homologue. In some embodiments, each one or more inactivated protease is independently selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2-related protein comprising an amino acid substitution or disruption of a DegP2 having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of an amino acid at a position corresponding to any one of positions 131, 134 and 236 of SEQ ID NO: 62 (or when excluding the 26 amino acid leader, positions 105, 108, and 210); an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr S210A; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr H105R; an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of any one or more amino acid at a position corresponding to any one of: 108-122, 146-152, and 217-234 of SEQ ID NO: 31. In some embodiments, the inactivated DegP, DegP-related protein, or DegP homologue has a substitution or disruption of any one or more amino acid at a position corresponding to any one

of: 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234 of SEQ ID NO: 31. In some embodiments, the inactivated DegP, DegP-related protein, or DegP homologue has a substitution or disruption of any one or more amino acid at a position corresponding to any one of: 116 (His), 120 (Asp), 122 (Asp) and 219 (Ser).

[0143] In some embodiments, an inactivated DegP, DegP-related protein, or DegP homologue comprises an amino acid substitution or disruption in the catalytic site of the corresponding DegP, DegP-related protein, or DegP homologue. Pallen and Wren, 1997, and Skorko-Glonek, J. et al., 1995, “Site-directed mutagenesis of the HtrA(DegP) serine protease, whose proteolytic activity is indispensable for *Escherichia coli* survival at elevated temperatures,” *Gene* 163:47-52, incorporated herein by reference, describe the catalytic site of DegP/HtrA and related proteins. In particular, amino acid residues S210 and H105 are critical for protease activity. The amino acid substitutions S210A and H105R eliminate proteolytic activity. Pallen and Wren describe the catalytic domain, including a “catalytic triad” of the amino acids His, Asp, and Ser. In *E. coli* Htr these critical residues occur at positions 131 (His), 134 (Asp) and 236 (Ser) (SEQ ID NO: 62, referring to numbering including leader sequence 1-26), or at respective positions 105, 108, and 210, when excluding the leader sequence. In *P. fluorescens* DegP2 (SEQ ID NO: 31), the catalytic triad

residues occur at positions 116 (His), 120 (Asp) and 219 (Ser), with numbering including leader sequence 1-27; excluding leader sequence, the catalytic triad residues occur at positions 89 (His), 93

(Asp), and 192 (Ser). One of skill in the art may identify the corresponding active site and catalytic triad in any DegP, DegP-related protein, or DegP homologue. In some embodiments, an inactivated DegP, DegP-related protein, or DegP homologue comprises an amino acid substitution or disruption of any one or more catalytic triad amino acid corresponding to any one of positions 116 (His), 120 (Asp) and 219 (Ser) of SEQ ID NO: 31. In some embodiments, an inactivated DegP, DegP-related protein, or DegP homologue comprises an amino acid substitution or disruption of any one or more catalytic triad amino acid corresponding to any one of positions 131 (His), 134 (Asp) and 236 (Ser) (SEQ ID NO: 62, referring to numbering including leader sequence 1-26), or at positions 105, 108, and 210, respectively, when excluding the leader sequence. In some embodiments, an inactivated DegP, DegP-related protein, or DegP homologue comprises an amino acid substitution or disruption of any one or more amino acid corresponding to any one of 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234 of SEQ ID NO: 31.

[0144] In some embodiments, the recombinant gram-negative host cell overexpresses 1 inactivated protease to 10 inactivated proteases. In some embodiments, the recombinant gram-negative host cell overexpresses 1 inactivated protease to 2 inactivated proteases, 1 inactivated protease to 3 inactivated proteases, 1 inactivated protease to 4 inactivated proteases, 1 inactivated protease to 5 inactivated proteases, 1 inactivated protease to 6 inactivated proteases, 1 inactivated protease to 7 inactivated proteases, 1 inactivated protease to 8 inactivated proteases, 1 inactivated protease to 9 inactivated proteases, 1 inactivated protease to 10 inactivated proteases, 2 inactivated proteases to 3 inactivated proteases, 2 inactivated proteases to 4 inactivated proteases, 2 inactivated proteases to 5 inactivated proteases, 2 inactivated proteases to 6 inactivated proteases, 2 inactivated proteases to 7 inactivated proteases, 2 inactivated proteases to 8 inactivated proteases, 2 inactivated proteases to 9 inactivated proteases, 2 inactivated proteases to 10 inactivated proteases, 3 inactivated proteases to 4 inactivated proteases, 3 inactivated proteases to 5 inactivated proteases, 3 inactivated proteases to 6 inactivated proteases, 3 inactivated proteases to 7 inactivated proteases, 3 inactivated proteases to 8 inactivated proteases, 3 inactivated proteases to 9 inactivated proteases, 3 inactivated proteases to 10 inactivated proteases, 4 inactivated proteases to 5 inactivated proteases, 4 inactivated proteases to 6 inactivated proteases, 4 inactivated proteases to 7 inactivated proteases, 4 inactivated proteases to 8 inactivated proteases, 4 inactivated proteases to 9 inactivated proteases, 4 inactivated proteases to 10 inactivated proteases, 5 inactivated proteases to 6 inactivated proteases, 5 inactivated proteases to 7 inactivated proteases, 5 inactivated proteases to 8 inactivated proteases, 5 inactivated proteases to 9 inactivated proteases, 5 inactivated proteases to 10 inactivated proteases, 6 inactivated proteases to 7 inactivated proteases, 6 inactivated proteases to 8 inactivated proteases, 6 inactivated proteases to 9 inactivated proteases, 6 inactivated proteases to 10 inactivated proteases, 7 inactivated proteases to 8 inactivated proteases, 7 inactivated proteases to 9 inactivated proteases, 7 inactivated proteases to 10 inactivated proteases, 8 inactivated proteases to 9 inactivated proteases, 8 inactivated proteases to 10 inactivated proteases, or 9 inactivated proteases to 10 inactivated proteases. In some embodiments, the recombinant gram-negative host cell overexpresses 1 inactivated protease, 2 inactivated proteases, 3 inactivated proteases, 4 inactivated proteases, 5 inactivated proteases, 6 inactivated proteases, 7 inactivated proteases, 8 inactivated proteases, 9 inactivated proteases, or 10 inactivated proteases. In some embodiments, the recombinant gram-negative host cell overexpresses at least 1 inactivated protease, 2 inactivated proteases, 3 inactivated proteases, 4 inactivated proteases, 5 inactivated proteases, 6 inactivated proteases, 7 inactivated proteases, 8 inactivated proteases, or 9 inactivated proteases. In some embodiments, the recombinant gram-negative host cell overexpresses at most 2 inactivated proteases, 3 inactivated proteases, 4

inactivated proteases, 5 inactivated proteases, 6 inactivated proteases, 7 inactivated proteases, 8 inactivated proteases, 9 inactivated proteases, or 10 inactivated proteases.

[0145] Protein Folding Modulators

[0146] In some embodiments, the one or more co-overexpressed protein is a protein folding modulator that improves the quality and/or yield of the recombinant protein of interest. Protein folding modulators, including chaperones, disulfide bond isomerases, and peptidyl-prolyl cis-trans isomerases (PPIases) are a class of proteins present in all cells that aid in the folding, unfolding and degradation of nascent polypeptides. An overexpressed protein folding modulator can be exogenously produced, e.g., from an expression construct on a plasmid. In some embodiments, a recombinant gram-negative host cell of the present invention overexpresses any one or more different protein folding modulator. In some embodiments, a recombinant gram-negative host cell of the present invention overexpresses 1 to 10 different protein folding modulators.

[0147] In some embodiments, a protein folding modulator is microbial. In some embodiments, a microbial protein folding modulator is from a bacterium, a mammal, a fungus (e.g., a yeast or a filamentous fungus), an arthropod (e.g., an arachnid or an insect), or a *Plasmodium*. In some embodiments, a bacterial protein folding modulator is from a gram-negative bacteria. In some embodiments, a mammalian protein folding modulator is from a rodent, e.g., a mouse, rat or hamster, e.g., a golden hamster. In some embodiments, a mammalian protein folding modulator is from a primate, e.g., an orangutan, a human, a horse, a pig, a bird, e.g., a flycatcher. In some embodiments, a gram-negative bacterial protein folding modulator is an *E. coli* or *Pseudomonas* folding modulator protein. In some embodiments, a protein folding modulator or chaperone is a *P. fluorescens* protein folding modulator. An overexpressed protein folding modulator may be any described in, e.g., U.S. Pat. No. 10,118,956, "Fusion Partners for Peptide Production" (e.g., as in Table 1), U.S. Pat. No. 9,580,719 (e.g., providing sequences for each folding modulator by RXF listed in Table 1 of U.S. Pat. No. 10,118,956), and U.S. Pat. No. 8,603,824, (e.g., Tables A to F therein). As used herein, RXF numbers are open reading frame numbers, and PROKKA numbers are designations determined using the Prokka tool as described by, e.g., Seemann, T., 2014, "Prokka: rapid prokaryotic genome annotation," *Bioinformatics* 30 (14): 2068–2069, incorporated herein by reference.

[0148] In some embodiments, a protein folding modulator is any known to those of skill in the art or described in the literature, e.g., in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts," 1997, ed. M. Gething, Melbourne University, Australia, incorporated herein by reference. In some embodiments, each one or more protein folding modulator is independently selected from a GroES/EL, DnaKJ, Clp, Hsp90, SecB, HSP70, HSP110/SSE,

HSP40 (DnaJ-related), GRPE-like, HSP90, CPN60, CPN10, cytosolic chaperone, HSP100, small HSP, calnexin, calreticulin, protein disulfide isomerase (PDI), thioredoxin-related protein, disulfide bond isomerase, protein disulfide isomerase, peptidyl-prolyl isomerase, cyclophilin PPIase, FK-506 binding protein, parvulin PPIase, individual chaperone, protein specific chaperone, or an intramolecular chaperone.

[0149] In some embodiments, an overexpressed folding modulator protein is a disulfide bond isomerase. In some embodiments, a disulfide bond isomerase is a gram-negative bacterial DsbA, DsbB, DsbC, DsbD, or DsbG. In some embodiments, a disulfide bond isomerase is selected from SEQ ID NOS: 60 (DsbC), 76 (putative cytoplasmic disulfide isomerase DsbA), 77 (DsbA), 78 (DsbB), 80 (DsbD), or 81 (DsbG). In some embodiments, an overexpressed folding modulator protein is a protein disulfide isomerase. In some embodiments, a protein disulfide isomerase is a PDIA6. In some embodiments, a PDIA6 has an amino acid sequence selected from SEQ ID NOS: 27 and 82-98. In some embodiments, an overexpressed protein folding modulator has an amino acid sequence selected from: SEQ ID NOS: 27, 57, 60, 76-78, and 80-98.

[0150] In some embodiments, the recombinant gram-negative host cell overexpresses 1 protein folding modulator to 10 protein folding modulators. In some embodiments, the recombinant gram-negative host cell overexpresses 1 protein folding modulator to 2 protein folding modulators, 1 protein folding modulator to 3 protein folding modulators, 1 protein folding modulator to 4 protein folding modulators, 1 protein folding modulator to 5 protein folding modulators, 1 protein folding modulator to 6 protein folding modulators, 1 protein folding modulator to 7 protein folding modulators, 1 protein folding modulator to 8 protein folding modulators, 1 protein folding modulator to 9 protein folding modulators, 1 protein folding modulator to 10 protein folding modulators, 2 protein folding modulators to 3 protein folding modulators, 2 protein folding modulators to 4 protein folding modulators, 2 protein folding modulators to 5 protein folding modulators, 2 protein folding modulators to 6 protein folding modulators, 2 protein folding modulators to 7 protein folding modulators, 2 protein folding modulators to 8 protein folding modulators, 2 protein folding modulators to 9 protein folding modulators, 2 protein folding modulators to 10 protein folding modulators, 3 protein folding modulators to 4 protein folding modulators, 3 protein folding modulators to 5 protein folding modulators, 3 protein folding modulators to 6 protein folding modulators, 3 protein folding modulators to 7 protein folding modulators, 3 protein folding modulators to 8 protein folding modulators, 3 protein folding modulators to 9 protein folding modulators, 3 protein folding modulators to 10 protein folding modulators, 4 protein folding modulators to 5 protein folding modulators, 4 protein folding modulators to 6 protein folding modulators, 4 protein folding

modulators to 7 protein folding modulators, 4 protein folding modulators to 8 protein folding modulators, 4 protein folding modulators to 9 protein folding modulators, 4 protein folding modulators to 10 protein folding modulators, 5 protein folding modulators to 6 protein folding modulators, 5 protein folding modulators to 7 protein folding modulators, 5 protein folding modulators to 8 protein folding modulators, 5 protein folding modulators to 9 protein folding modulators, 5 protein folding modulators to 10 protein folding modulators, 6 protein folding modulators to 7 protein folding modulators, 6 protein folding modulators to 8 protein folding modulators, 6 protein folding modulators to 9 protein folding modulators, 6 protein folding modulators to 10 protein folding modulators, 7 protein folding modulators to 8 protein folding modulators, 7 protein folding modulators to 9 protein folding modulators, 7 protein folding modulators to 10 protein folding modulators, 8 protein folding modulators to 9 protein folding modulators, 8 protein folding modulators to 10 protein folding modulators, or 9 protein folding modulators to 10 protein folding modulators. In some embodiments, the recombinant gram-negative host cell overexpresses 1 protein folding modulator, 2 protein folding modulators, 3 protein folding modulators, 4 protein folding modulators, 5 protein folding modulators, 6 protein folding modulators, 7 protein folding modulators, 8 protein folding modulators, 9 protein folding modulators, or 10 protein folding modulators. In some embodiments, the recombinant gram-negative host cell overexpresses at least 1 protein folding modulator, 2 protein folding modulators, 3 protein folding modulators, 4 protein folding modulators, 5 protein folding modulators, 6 protein folding modulators, 7 protein folding modulators, 8 protein folding modulators, or 9 protein folding modulators. In some embodiments, the recombinant gram-negative host cell overexpresses at most 2 protein folding modulators, 3 protein folding modulators, 4 protein folding modulators, 5 protein folding modulators, 6 protein folding modulators, 7 protein folding modulators, 8 protein folding modulators, 9 protein folding modulators, or 10 protein folding modulators.

[0151] Related Proteins

[0152] Prior to being modified in accordance with the present invention, e. g., to introduce a mutation in a gene to result in a deficient protein activity, a bacterial host cell may have multiple genes that encode the same protein, or that encode multiple proteins having the same or similar activity, e.g., a protease activity or autolytic factor activity. Under these circumstances, the protein deficiency of the recombinant bacterial host cell may result from mutation of more than one gene.

[0153] Two different gram-negative bacterial host cells, e.g., host cells of different genera or species, may have multiple related proteins. These related proteins may have similar sequences, structures, functions, and/or activities. Under these circumstances, a deficient protein activity of

the first host cell and a deficient protein activity of the second host cell may result from mutations of genes having a high level of amino acid sequence similarity or identity. Between different host cells (e.g., of different species), and within the same host cell, certain such proteins are described in the literature as homologues based on the knowledge or assumption of an ancestral link.

[0154] In the context of the present invention, regardless of an ancestral link, one of skill in the art may identify two proteins as related proteins (within the same host cell, e.g., the same host cell species, or between two different host cells), using methods known in the art and described herein.

[0155] In some embodiments, as referred to herein, related proteins, e.g., related proteases or related autolytic factors, have defined amino acid sequence similarity or identity. It is understood that any amino acid sequence similarity or identity range provided elsewhere herein may be replaced with a narrower range falling within that range, and that any minimum amino acid sequence similarity or identity provided herein may be replaced with a higher minimum. In some embodiments, a “related protein” as used herein may have an amino acid sequence similarity or identity, active/catalytic site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of about 30% to about 100%. Sequence similarity or identity of nucleic acid or amino acid sequences as described herein may be determined by methods known to those of skill in the art. In some embodiments, amino acids are similar with regard to polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. Thus, a similar amino acid may be an amino acid identified as suitable for a conservative amino acid substitution, e.g., as described in the literature and readily identified by methods known to those of skill in the art, for example, as shown in **Table 2**, listing conservative amino acid substitutions. In some embodiments, a similar amino acid is an amino acid listed in **Table 2**, second column (headed “I. Conservative Substitutions”) in the row corresponding to the original amino acid. In some embodiments, a similar amino acid is an amino acid listed in **Table 2**, third column (headed “II. Alternative Substitutions”) in the row corresponding to the original amino acid.

[0156] In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of about 30% to about 35%, about 30% to about 40%, about 30% to about

45%, about 30% to about 50%, about 30% to about 55%, about 30% to about 60%, about 30% to about 65%, about 30% to about 70%, about 30% to about 80%, about 30% to about 90%, about 30% to about 100%, about 35% to about 40%, about 35% to about 45%, about 35% to about 50%, about 35% to about 55%, about 35% to about 60%, about 35% to about 65%, about 35% to about 70%, about 35% to about 80%, about 35% to about 90%, about 35% to about 100%, about 40% to about 45%, about 40% to about 50%, about 40% to about 55%, about 40% to about 60%, about 40% to about 65%, about 40% to about 70%, about 40% to about 80%, about 40% to about 90%, about 40% to about 100%, about 45% to about 50%, about 45% to about 55%, about 45% to about 60%, about 45% to about 65%, about 45% to about 70%, about 45% to about 80%, about 45% to about 90%, about 45% to about 100%, about 50% to about 55%, about 50% to about 60%, about 50% to about 65%, about 50% to about 70%, about 50% to about 80%, about 50% to about 90%, about 50% to about 100%, about 55% to about 60%, about 55% to about 65%, about 55% to about 70%, about 55% to about 80%, about 55% to about 90%, about 55% to about 100%, about 60% to about 65%, about 60% to about 70%, about 60% to about 80%, about 60% to about 90%, about 60% to about 100%, about 65% to about 70%, about 65% to about 80%, about 65% to about 90%, about 65% to about 100%, about 70% to about 80%, about 70% to about 90%, about 70% to about 100%, about 80% to about 90%, about 80% to about 100%, or about 90% to about 100%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 80%, about 90%, or about 100%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of at least about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 80%, or about 90%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of at most about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 80%, about 90%, or about 100%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of about 45% to about 50%, about 45% to about 55%, about 45% to about 60%, about 45% to about 65%, about 45% to about 70%, about 45% to about 75%, about 45% to about 80%, about 45% to about 85%, about 45% to about 90%, about 45% to about 95%, about 45% to about 100%, about 50% to about 55%, about 50% to about 60%, about 50% to about 65%, about 50% to about 70%, about 50% to about 75%, about

50% to about 80%, about 50% to about 85%, about 50% to about 90%, about 50% to about 95%, about 50% to about 100%, about 55% to about 60%, about 55% to about 65%, about 55% to about 70%, about 55% to about 75%, about 55% to about 80%, about 55% to about 85%, about 55% to about 90%, about 55% to about 95%, about 55% to about 100%, about 60% to about 65%, about 60% to about 70%, about 60% to about 75%, about 60% to about 80%, about 60% to about 85%, about 60% to about 90%, about 60% to about 95%, about 60% to about 100%, about 65% to about 70%, about 65% to about 75%, about 65% to about 80%, about 65% to about 85%, about 65% to about 90%, about 65% to about 95%, about 65% to about 100%, about 70% to about 75%, about 70% to about 80%, about 70% to about 85%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 75% to about 80%, about 75% to about 85%, about 75% to about 90%, about 75% to about 95%, about 75% to about 100%, about 80% to about 85%, about 80% to about 90%, about 80% to about 95%, about 80% to about 100%, about 85% to about 90%, about 85% to about 95%, about 85% to about 100%, about 90% to about 95%, about 90% to about 100%, or about 95% to about 100%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 100%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of at least about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, related proteins have amino acid sequence similarity or identity, active site amino acid sequence similarity or identity, or allosteric region amino acid sequence similarity or identity, of at most about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 100%.

[0157] Related proteins can be homologues, or may not be homologues. In some embodiments, a related protein that is a homologue of a given protein is identified using the protein sequence as a query sequence in a PSI-BLAST (Position-Specific Iterative basic Local Alignment Search Tool) search of all nonredundant (nr) protein sequences from the host cell (e.g., *E. coli*) proteome sequences deposited in the NCBI database, at default settings. PSI-BLAST search methods are known to those of skill in the art and have been described by, e.g., Bhagwat, M., and Aravind, L., 2007, "PSI-BLAST Tutorial," Ch. 10 in *Comparative Genomics: Volumes 1 and 2*, Bergman NH, ed., Totowa (NJ): Humana Press, incorporated herein by reference. Approaches to identifying homologues is described in the literature, e.g., by Pearson, W.R., 2014, "BLAST and

FASTA similarity searching for multiple sequence alignment,” *Methods Mol. Biol.* 1079:75-101, incorporated herein by reference in its entirety.

[0158] Nucleic acid and amino acid sequence similarity identity may be determined according to any suitable method known in the art, including but not limited to those described herein. For example, alignments and searches for similar sequences can be performed using the U.S. National Center for Biotechnology Information (NCBI, Bethesda, MD) program, MegaBLAST. Use of this program with options for percent identity set at, for example, 70% for amino acid sequences, or set at, for example, 90% for nucleotide sequences, will identify those sequences with 70%, or 90%, or greater sequence identity to the query sequence. Other software known in the art is also available for aligning and/or searching for similar sequences, e.g., sequences at least 70% or 90% identical to an information string containing a secretion signal sequence herein. For example, sequence alignments for comparison to identify sequences at least 70% or 90% identical to a query sequence is often performed by use of, e.g., the GAP, BESTFIT, BLAST, FASTA, and TFASTA programs available in the GCG Sequence Analysis Software Package (available from the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705), with the default parameters as specified therein, plus a parameter for the extent of sequence identity set at the desired percentage. Also, for example, the CLUSTAL program (available in the PC/Gene software package from Intelligenetics, Mountain View, CA.) may be used.

[0159] These and other sequence alignment methods are well known in the art and may be conducted by manual alignment, by visual inspection, or by manual or automatic application of a sequence alignment algorithm, such as any of those embodied by the above-described programs. Various useful algorithms include, e.g.: the similarity search method described in W. R. Pearson & D. J. Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444-48 (April 1988); the local homology method described in T. F. Smith & M. S. Waterman, in *Adv. Appl. Math.* 2:482-89 (1981) and in *J. Molec. Biol.* 147:195-97 (1981); the homology alignment method described in S. B. Needleman & C. D. Wunsch, *J. Molec. Biol.* 48(3):443-53 (March 1970); and the various methods described, e.g., by W. R. Pearson, in *Genomics* 11(3):635-50 (November 1991); by W. R. Pearson, in *Methods Molec. Biol.* 24:307-31 and 25:365-89 (1994); and by D. G. Higgins & P. M. Sharp, in *Comp. Appl'ns in Biosci.* 5:151-53 (1989) and in *Gene* 73(1):237-44 (15 Dec. 1988).

[0160] GAP Version 10, which uses the algorithm of Needleman and Wunsch (1970) *supra*, can be used to determine sequence identity or similarity using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity or % similarity for an amino acid sequence using

GAP weight of 8 and length weight of 2, and the BLOSUM62 scoring program. Equivalent or similar programs may also be used as will be understood by one of skill in the art. For example, a sequence comparison program can be used that, for any two sequences in question, generates an alignment having identical nucleotide residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10. In embodiments, the sequence comparison is performed across the entirety of the query or the subject sequence, or both.

[0161] Mutations Resulting in a Deficiency of a Recombinant Host Cell Protein or in an Overexpressed Inactivated Protease

[0162] A recombinant bacterial host cell of the invention having a deficient protein activity can be generated by altering one or more genes encoding a protein having the protein activity, by any known method. A “deficient” protein activity or “deficiency” in a protein activity as used throughout this description, may include a partial deficiency, a substantial deficiency, or a complete deficiency. A “deficient” protein activity or “deficiency” in a protein activity as used throughout this description may include a reduction in, or elimination of, the protein activity. In some embodiments, the recombinant host cell protein activity is accordingly deficient in the host cell as compared with a control cell. In some embodiments, a control cell is a corresponding host cell that has wild-type activity of the protein. In some embodiments, a control cell is a corresponding wild-type cell. In some embodiments, a control cell has wild-type activity of the protein but has other differences relative to a wild-type cell. The recombinant host cell of the invention may be modified by any suitable means, e.g., as described herein, to reduce or eliminate the activity of protein. A recombinant bacterial host cell of the invention may also overexpress an inactivated protease, as described herein. In some embodiments, the overexpressed inactivated protease is partially inactivated, substantially inactivated, or fully inactivated with regard to the protease activity. In some embodiments, the overexpressed inactivated protease is partially inactivated, substantially inactivated, or fully inactivated with regard to the protease activity, and active with respect to another property, e.g., a chaperone activity. In some embodiments, the inactivated protease is inactivated by mutation, e.g., by mutation of a gene encoding the active protease (having protease activity).

[0163] In some embodiments, the deficient or reduced protein activity of the recombinant host cell results from a mutation that causes an amino acid change or other disruption, e.g., by amino acid substitution, deletion of one or more amino acid, insertion of one or more amino acid, or protein truncation. In some embodiments, the mutation is an inactivating mutation. In some embodiments, the mutation is a partially-inactivating mutation. In some embodiments, a deficiency in the activity of a protein, e.g., a protease or autolytic factor, results from one or more

mutation independently selected from (i) a complete gene deletion (gene knockout), (ii) a partial gene deletion, (iii) a missense mutation, (iv) a nonsense mutation, (v) a frameshift mutation, (vi) an insertion, and (vii) any combination of (ii), (iii), (iv), (v) and (vi). In some embodiments, an overexpressed inactivated protease is inactivated by one or more mutation independently selected from (ii) a partial gene deletion, (iii) a missense mutation, (iv) a nonsense mutation, (v) a frameshift mutation, (vi) an insertion, and (vii) any combination of (ii), (iii), (iv), (v) and (vi). In some embodiments, the mutation resulting in a deficient protein activity or an inactivated protease is in a coding region of a gene encoding the protein or inactivated protease. In some embodiments, the mutation resulting in a deficient protein activity is in a non-coding region of the gene encoding the protein. In some embodiments, the non-coding region of the gene is a regulatory region. In some embodiments, the mutation in the regulatory region of the gene disrupts a regulatory element that is required for production of the protein, for example, an element required for transcription of the corresponding RNA, or translation of the mRNA into protein. For example, a noncoding region regulatory element can be a promoter, enhancer, regulatory protein binding site, ribosome binding site, or any other regulatory element as known to those of skill in the art.

[0164] In some embodiments, a mutation disrupts a critical site in a protein to result in a deficient protein in the recombinant host cell, or an inactivated overexpressed protease, e.g., by changing or deleting one or more amino acids at a protease active site. In some embodiments, a mutation disrupts an allosteric region of the protein, e.g., by changing one or more amino acids in an allosteric region. An allosteric region may be a region that interacts with another region to form an active protein conformation. In some embodiments, a mutation results in the substitution of an amino acid with any other amino acid. In some embodiments, the substitution is a non-conservative amino acid substitution. A non-conservative amino acid substitution can be readily selected by one of skill in the art. **Table 2** provides examples of conservative amino acid substitutions (column I) and alternative conservative amino acid substitutions (II). In some embodiments, a non-conservative substitution of an original amino acid (e.g., the amino acid in the wild-type protein) is a substitution with any amino acid not listed in (I) for the original amino acid. In some embodiments, a non-conservative substitution of an original amino acid is any amino acid not listed in (II) for the original amino acid. In some embodiments, a non-conservative amino acid substitution is any amino acid not listed in either (I) or (II) for the original amino acid.

[0165] **Table 2.**

Amino Acid	I. Conservative Substitutions	II. Alternative Substitutions
Ala	Gly, Ile, Leu, Val	any aliphatic amino acid or derivative thereof (Ala , Gly, Ile, Leu, Val) any hydrophobic amino acid or derivative thereof (Ala , Gly, Ile, Leu, Met, Phe, Pro, Val)
Arg	His, Lys	any basic amino acid or derivative thereof (Arg , His, Lys) any charged amino acid or derivative thereof (Asp, Arg , Glu, Lys) any basic amino acid with an electrically charged sidechain or derivative thereof (Arg , His, Lys)
Asn	Asp, Gln, Glu	any acidic amino acid or derivative thereof, or any amide of any acidic amino acid or derivative thereof (Asn , Asp, Gln, Glu) any polar amino acid or derivative thereof (Asn , Cys, Gln, His, Ser, Thr, Trp, Tyr)
Asp	Asn, Gln, Glu	any acidic amino acid or derivative thereof, or any amide of any acidic amino acid or derivative thereof (Asn, Asp , Gln, Glu) any charged amino acid or derivative thereof (Asp , Arg, Glu, Lys) any polar neutral amino acid or derivative thereof (Asp , Cys, Gln, Ser, Thr) any acidic amino acid with an electrically charged sidechain or derivative thereof (Asp , Glu)
Cys	Met, Sec, Ser, Thr	any hydroxyl or sulfur/selenium-containing amino acid or derivative thereof (Cys , Sec, Ser, Met, Thr)

		any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr) any polar neutral amino acid or derivative thereof (Asp, Cys, Gln, Ser, Thr)
Gln	Asn, Asp, Glu	any acidic amino acid or derivative thereof, or any amide of any acidic amino acid or derivative thereof (Asn, Asp, Gln, Glu) any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr) any polar neutral amino acid or derivative thereof (Asp, Cys, Gln, Ser, Thr) any acidic amino acid with an electrically charged sidechain or derivative thereof (Asp, Glu)
Glu	Asn, Asp, Gln	any acidic amino acid or derivative thereof, or any amide of any acidic amino acid or derivative thereof (Asn, Asp, Gln, Glu) any charged amino acid or derivative thereof (Asp, Arg, Glu, Lys)
Gly	Ala, Ile, Leu, Val	any aliphatic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Val) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Met, Phe, Pro, Val)
His	Arg, Lys	any basic amino acid or derivative thereof (Arg, His, Lys) any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr) any basic amino acid with an electrically charged sidechain or derivative thereof (Arg, His, Lys)

Ile	Ala, Gly, Leu, Val	any aliphatic amino acid or derivative thereof (Ala, Gly, Ile , Leu, Val) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile , Leu, Met, Phe, Pro, Val)
Leu	Ala, Gly, Ile, Val	any aliphatic amino acid or derivative thereof (Ala, Gly, Ile, Leu , Val) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile, Leu , Met, Phe, Pro, Val)
Lys	Arg, His	any basic amino acid or derivative thereof (Arg, His, Lys) any charged amino acid or derivative thereof (Asp, Arg, Glu, Lys) any basic amino acid with an electrically charged sidechain or derivative thereof (Arg, His, Lys)
Met	Cys, Sec, Ser, Thr	any hydroxyl or sulfur/selenium-containing amino acid or derivative thereof (Cys, Sec, Ser, Met , Thr) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Met , Phe, Pro, Val)
Phe	Trp, Tyr	any aromatic amino acid or derivative thereof (Phe , Trp, Tyr) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Met, Phe , Pro, Val)
Pro		any cyclic amino acid or derivative thereof (Pro) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Met, Phe, Pro , Val)
Ser	Cys, Met, Sec, Thr	any hydroxyl or sulfur/selenium-containing amino acid or derivative thereof

		(Cys, Sec, Ser, Met, Thr) any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr) any polar neutral amino acid or derivative thereof (Asp, Cys, Gln, Ser, Thr)
Thr	Cys, Met, Sec, Ser	any hydroxyl or sulfur/selenium-containing amino acid or derivative thereof (Cys, Sec, Ser, Met, Thr) any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr) any polar neutral amino acid or derivative thereof (Asp, Cys, Gln, Ser, Thr)
Trp	Phe, Tyr,	any aromatic amino acid or derivative thereof (Phe, Trp, Tyr) any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr)
Tyr	Phe, Trp	any aromatic amino acid or derivative thereof (Phe, Trp, Tyr) any polar amino acid or derivative thereof (Asn, Cys, Gln, His, Ser, Thr, Trp, Tyr)
Val	Ala, Gly, Ile, Leu	any aliphatic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Val) any hydrophobic amino acid or derivative thereof (Ala, Gly, Ile, Leu, Met, Phe, Pro, Val)

[0166] Recombinant Proteins of Interest

[0167] The present invention provides gram-negative bacterial host cells and methods for their use to produce recombinant proteins of interest of high quality and at high yield. In some embodiments, a recombinant protein of interest produced using the described host cells and

methods is a protein susceptible to degradation when recombinantly expressed in gram-negative bacterial host cell. In some embodiments the recombinant protein of interest is degraded in a host cell that produces a wild-type, or functional, tail-specific protease, and is observed to be less degraded in a host cell deficient in tail-specific protease activity. As described herein, a recombinant protein of interest may be produced by the recombinant gram-negative bacterial host cell from one or more expression plasmid or vector comprising nucleic acid expression constructs that encode the recombinant protein of interest and from which the recombinant protein can be expressed.

[0168] Extensive sequence information required for molecular genetics and genetic engineering techniques is widely publicly available. Access to complete nucleotide sequences of mammalian, as well as human, genes, cDNA sequences, amino acid sequences and genomes are often obtained from GenBank at the website www.ncbi.nlm.nih.gov/Entrez. Additional information may be obtained from GeneCards, an electronic encyclopedia integrating information about genes and their products and biomedical applications, from the Weizmann Institute of Science Genome and Bioinformatics. Nucleotide sequence information may be obtained from the EMBL Nucleotide Sequence Database or the DNA Databank or Japan (DDBJ). Additional sources of information on amino acid sequences include Georgetown's protein information resource website and Swiss-Prot.

[0169] In some embodiments, the protein of interest is a mammalian protein or polypeptide or derived from a mammalian protein or polypeptide. The terms “protein” and “polypeptide” are used interchangeably herein, unless the context indicates otherwise. In embodiments, the protein of interest is a human protein or derived from a human protein. In embodiments, the protein of interest is a prokaryotic protein or derived from a prokaryotic protein. In embodiments, the protein of interest is a microbial protein or derived from a microbial protein. In embodiments, the protein of interest is a bacterial protein or derived from a bacterial protein. In some embodiments, the recombinant protein of interest is human, murine, rat, rabbit, guinea pig, camelid, shark, avian, yeast, fungal, gram-negative bacterial, or gram-positive bacterial, or derived therefrom.

[0170] In some embodiments, a recombinant protein of interest produced using the compositions and methods of the present invention is selected from: an antibody, antibody fragment, or derivative of an antibody or antibody fragment (antibody, antibody fragment, or derivative thereof); an antibody-based drug, a non-antibody binding protein (e.g., an antibody mimetic, including, but not limited to, an alphabody, an iBody, an affibody, an affilin, an affitin, or an anticalin), a reagent protein; a vaccine antigen; a therapeutic protein or enzyme; non-natural protein; a pathogen protein or derivative thereof; a microbial toxin, a lipoprotein; an extracellular

receptor or ligand; a protease; a kinase; a blood protein; a chemokine; a cytokine; a bone morphogenic protein; an anticoagulant; a blood factor; a bone morphogenetic protein; an engineered protein scaffold; an enzyme, e.g., a biocatalytic enzyme; a growth factor; an interferon; an interleukin; a thrombolytic agent; a hormone; and a TGF-beta family member protein.

[0171] In some embodiments, a recombinant protein of interest produced using the compositions and methods of the present invention is mammalian, rodent, avian, Chondrichthyes, fungal, or bacterial. In some embodiments, a recombinant protein of interest is human, murine, rat, rabbit, guinea pig, camelid, shark, chicken, yeast, fungal, gram-negative bacterial, or gram-positive bacterial. In some embodiments, the recombinant protein of interest is native to the recombinant gram-negative bacterial host cell. In some embodiments, the recombinant protein of interest is heterologous to the recombinant gram-negative bacterial host cell, that is, the protein of interest is derived from an organism other than the expression host cell. In some embodiments, a recombinant protein of interest produced is a difficult-to-express recombinant protein, e.g., a protein that undergoes rapid proteolytic degradation by intracellular bacterial proteases, including a protein having an N-terminus that is vulnerable to degradation, and a protein that typically is produced in insoluble form in microbial or bacterial expression systems.

[0172] In some embodiments, an antibody, antibody fragment, or derivative of an antibody or antibody fragment (antibody, antibody fragment, or derivative thereof is selected from: a monoclonal antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; modified antibody, a bispecific antibody, a chimeric antibody; a diabody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a tribody; an intrabody; a nanobody; a small modular immunopharmaceutical (SMIP); an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody, an avian antibody (e.g., a chicken antibody), a VHH-containing antibody; a F(ab); a F(ab)[']; F(ab)[']₂; scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment (e.g., generated by reducing the hinge region disulfide bonds of IgG); an Fc fusion protein (e.g., comprising the Fc domain of IgG fused together with a protein or peptide of interest); a domain antibody; a VL; a VNAR; a VH; a VHH; or any other antibody fragment described in the art, e.g., in U.S. Pat. No. 5,648,237, "Expression of Functional Antibody Fragments," incorporated by reference herein in its entirety. In some embodiments, the VHH-containing antibody is a VHH concatenated antibody. In some embodiments, an antibody or antibody fragment derived from a non-human animal species is humanized.

[0173] In some embodiments, an antibody, antibody fragment, or derivative thereof produced using the compositions and methods of the present invention is a therapeutic antibody, antibody fragment, or derivative thereof. In some embodiments, the therapeutic antibody, antibody fragment, or derivative thereof binds to a target selected from: a cytokine; a chemokine; a drug; a cell-surface protein, e.g., a receptor, cell-surface marker, pathogen surface-protein, etc.; a growth factor; a growth factor receptor; immune checkpoint molecule, and a blood factor. In some embodiments, the cytokine is TNF-alpha. In some embodiments, the drug is a platelet-aggregation inhibitor. In some embodiments, the platelet-aggregation inhibitor is ticagrelor.

[0174] In some embodiments, the recombinant protein of interest is an antibody fragment selected from a Fab, Fab', or F(ab')₂. A Fab comprises one constant region domain and one variable region domain of each of the heavy and the light chain, and lacks the antibody hinge region. In some embodiments, the recombinant protein of interest is a Fab'. A Fab' also comprises one constant region domain and one variable region domain of each of the heavy and the light chain, and also comprises the antibody hinge region of the heavy chain, and thereby has free sulfhydryls. A F(ab')₂ comprises two antigen binding regions, each having one constant region domain and one variable region domain of each of the heavy and the light chains, and both heavy chains having the hinge region. This allows disulfide bonding to join the two binding regions.

[0175] In some embodiments, the recombinant protein of interest is a Fab' that binds to a target selected from: Carcinoembryonic antigen (CEA); CD22; fibrin II, beta chain; TNF-alpha; and NCA-90 (granulocyte antigen). In some embodiments, the Fab' is selected from: Arcitumomab; Bectumomab; Biciromab; the Fab' moiety of Certolizumab pegol; and Sulesomab. In some embodiments, the recombinant protein of interest is a Fab that binds to a target selected from: EpCAM, Complement factor D (CFD), C242 antigen, 5T4, human scatter factor receptor kinase, VEGF-A, and integrin α IIb β 3. In some embodiments, the Fab is selected from: Abciximab; Abrezekimab; Anatumomab mafenatox; Citatuzumab bogatox; Lampalizumab; Nacolomab tafenatox; Naptumomab estafenatox; Nofetumomab merpentan; Onartuzumab; Ranibizumab; Tadocizumab; and Telimomab aritox. In some embodiments, the recombinant protein of interest is a F(ab')₂ that binds to a target selected from: TNF-alpha; VEGFR2; ITGB2 (CD18); and CA-125. In some embodiments, the F(ab')₂ is selected from: Afelimomab; Alacizumab pegol; Dorlimomab aritox; Erlizumab; and Igovomab.

[0176] In some embodiments, a Fab' that binds to human tumor necrosis factor alpha (human TNF-alpha) is the Fab' moiety of certolizumab. Certolizumab is a recombinant, humanized antibody Fab' fragment, with specificity for human TNF-alpha, conjugated to an approximately 40kDa polyethylene glycol (PEG2MAL40K). Certolizumab is approved in the United States

under the name Cimzia® for treatment of autoimmune conditions including Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, non-radiographic axial spondyloarthritis, and moderate to severe plaque psoriasis, as described in detail in the Cimzia Prescribing Information (Cimzia Prescribing Information, revised 09/2019, incorporated by reference in its entirety). In some embodiments, the recombinant gram-negative bacterial host cell and related methods described herein are used to produce a recombinant Fab' that binds to human TNF-alpha, for use in the treatment of Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, non-radiographic axial spondyloarthritis, and moderate to severe plaque psoriasis.

[0177] In some embodiments, a recombinant protein of interest is expressed in the recombinant gram-negative bacterial host cell from one or more expression vector, each comprising one or more expression constructs, and each expression construct comprising a nucleic acid sequence for expressing and encoding the recombinant protein of interest. As understood by one of skill in the art, one or more expression construct may be included in a single expression vector, as required to encode each polypeptide chain comprised by the recombinant protein of interest. In some embodiments, a recombinant gram-negative bacterial host cell comprises at least two expression vectors, each comprising one or more expression construct as needed depending upon the recombinant protein of interest. As understood by those of skill in the art, an antibody, antibody fragment, or derivative thereof may be comprised of one or more polypeptides produced from one or more expression constructs. For example, an antibody may be comprised of four polypeptides: two identical heavy chains and two identical light chains, encoded by at least two genes. An antibody may be comprised of two non-identical heavy chains and two non-identical light chains, encoded by at least four different genes. An antibody fragment may be, e.g., comprised of one heavy chain and one light chain (e.g., a Fab or Fab'), two heavy chains and two light chains (e.g., a F(ab')₂), one heavy chain (e.g., a VHH, a V_H, or a V_L), or a single polypeptide comprising both V_H and V_L (scFv). In some embodiments, one or more expression vector in a recombinant host cell may include multiple copies of the same expression construct.

[0178] In some embodiments, the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least one nucleic acid sequence encoding a heavy chain, at least one nucleic acid sequence encoding a light chain, or both, wherein the heavy chain is full-length or a heavy chain fragment, and the light chain is full-length or a light chain fragment. In some embodiments, the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least two nucleic acid sequences, each encoding a heavy chain. In some embodiments, the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises a nucleic acid sequence

encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from the same mRNA transcript. In some embodiments, the at least one expression construct encoding the antibody, an antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from different mRNA transcripts. In some embodiments, each heavy chain-encoding nucleic acid sequence and each light chain-encoding nucleic acid sequence is individually operably linked to an independently selected nucleic acid sequence encoding a periplasmic secretion signal.

[0179] Further, as described herein, a recombinant gram-negative host cell of the invention may comprise one or more expression vector that contains one or more expression construct for production of a co-overexpressed protein, e.g., an inactivated protease, folding modulator, chaperone, or any combination thereof. A recombinant protein of interest and a co-overexpressed protein expression may be expressed from the same expression vector. In some embodiments, a recombinant protein of interest and a co-overexpressed protein are co-transcribed, from the same promoter. In some embodiments, a recombinant protein of interest and a co-overexpressed protein are transcribed from different promoters. A recombinant protein of interest and a co-overexpressed protein may be expressed from different expression vectors in the recombinant host cell.

[0180] In some embodiments, a recombinant gram-negative bacterial host cell comprises 1 to 5 expression vectors. In some embodiments, each expression vector comprises 1 to 5 expression constructs. In some embodiments, the expression constructs each encode a different protein. In some embodiments, more than one expression construct present in the expression vector(s) encodes the same protein.

[0181] Methods for Producing a Recombinant Protein

[0182] The present invention includes methods for producing a recombinant protein of interest using the recombinant gram-negative bacterial host cells described herein. The compositions and methods of the invention can be used to produce a recombinant protein of interest of high quality, at high yield, or both. A high quality recombinant protein of interest can be soluble, active, intact, or any combination thereof. In some embodiments, the compositions and methods of the invention are used to produce a recombinant protein that is soluble, active, intact, present at high yield, or any combination thereof.

[0183] In some embodiments, a method for producing a recombinant protein of interest comprises: recovering the recombinant protein of interest from a recombinant gram-negative bacterial host cell as set forth herein, wherein the recombinant gram-negative host cell has been cultured under suitable fermentation conditions, wherein the recombinant gram-negative host cell

has been transformed with at least one expression vector encoding the recombinant protein of interest. In some embodiments, recovery of the recombinant protein of interest from the recombinant gram-negative bacterial host cell comprises at least one purification step. In some embodiments, the yield and/or quality of the recovered recombinant protein of interest is measured. In some embodiments, the yield and/or quality of the recovered recombinant protein of interest is compared with that recovered from a control cell.

[0184] Production and evaluation of a recombinant protein of interest using the inventive gram-negative bacterial host cells as described herein may be carried out as set forth herein, in combination with known tools and methods for producing recombinant proteins in bacterial host cells.

[0185] Gram-Negative Bacterial Host Cells

[0186] Gram-negative bacterial host cells of the present invention include *Pseudomonads* (i.e., host cells in the order *Pseudomonadales*) and related bacterial organisms known in the art, e.g., *Escherichia*, *Erwinia*, *Salmonella*, *Shigella*, *Moraxella*, *Helicobacter*, *Legionella*, *Neisseria*, *Haemophilus*, *Acinetobacter*, *Xylella*, *Bacteroides*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Proteus*, *Serratia*, *Shigella*, *Yersinia* and *Vibrio*, and including any species or subspecies, including but not limited to *P. fluorescens*, *P. aeruginosa*, *P. putida*, *E. coli*, *E. chrysanthemi*, *S. typhimurium*, *Helicobacter pylori*, *L. pneumophila*, *N. meningitidis*, *N. gonorrhoeae*, *Haemophilus influenzae*, *V. cholerae*, *X. fastidiosa*, and *A. baylyi*.

[0187] In some embodiments, the *Pseudomonad* host cell is *Pseudomonas fluorescens*.

[0188] In embodiments, the host cell is of the order *Pseudomonadales* (referred to herein as a “*Pseudomonad*.” Where the host cell is of the order *Pseudomonadales*, it may be a member of the family *Pseudomonadaceae*, including the genus *Pseudomonas*.

[0189] Gamma Proteobacterial hosts include members of the species *Escherichia coli* and members of the species *Pseudomonas fluorescens*. Other *Pseudomonas* organisms may also be useful. *Pseudomonads* and closely related species include Gram-negative Proteobacteria Subgroup 1, which include the group of Proteobacteria belonging to the families and/or genera described as “Gram-Negative Aerobic Rods and Cocci” by R. E. Buchanan and N. E. Gibbons (eds.), *Bergey's Manual of Determinative Bacteriology*, pp. 217-289 (8th ed., 1974) (The Williams & Wilkins Co., Baltimore, Md., USA), all are incorporated by reference herein in its entirety. **Table 3** presents these families and genera of organisms.

Table 3: Families and Genera (“Gram-Negative Aerobic Rods and Cocci,” Bergey’s, 1974)

Family I. <i>Pseudomonaceae</i> <i>Gluconobacter</i>	<i>Pseudomonas</i> <i>Xanthomonas</i> <i>Zoogloea</i>
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Family II. <i>Azotobacteraceae</i> <i>Azomonas</i>	<i>Azotobacter</i> <i>Beijerinckia</i> <i>Derxia</i>
Family III. <i>Rhizobiaceae</i> <i>Agrobacterium</i>	<i>Rhizobium</i>
Family IV. <i>Methylomonadaceae</i> <i>Methylococcus</i>	<i>Methylomonas</i>
Family V. <i>Halobacteriaceae</i> <i>Halobacterium</i>	<i>Halococcus</i>
Other Genera <i>Acetobacter</i>	<i>Alcaligenes</i> <i>Bordetella</i> <i>Brucella</i> <i>Francisella</i> <i>Thermus</i>

[0190] *Pseudomonas* and closely related bacteria are generally part of the group defined as “Gram(-) Proteobacteria Subgroup 1” or “Gram-Negative Aerobic Rods and Cocci” (Buchanan and Gibbons (eds.) (1974) *Bergey's Manual of Determinative Bacteriology*, pp. 217-289). *Pseudomonas* host strains are described in the literature, e.g., in U.S. Pat. No. 9,458,487 and U.S. Pat. No. 9,453,251, both entitled “Expression of mammalian proteins in *Pseudomonas fluorescens*,” each incorporated by reference herein.

[0191] “Gram-negative Proteobacteria Subgroup 1” also includes Proteobacteria that would be classified in this heading according to the criteria used in the classification. The heading also includes groups that were previously classified in this section but are no longer, such as the genera *Acidovorax*, *Brevundimonas*, *Burkholderia*, *Hydrogenophaga*, *Oceanimonas*, *Ralstonia*, and *Stenotrophomonas*, the genus *Sphingomonas* (and the genus *Blastomonas*, derived therefrom), which was created by regrouping organisms belonging to (and previously called species of) the genus *Xanthomonas*, the genus *Acidomonas*, which was created by regrouping organisms belonging to the genus *Acetobacter* as defined in Bergey (1974). In addition hosts can include cells from the genus *Pseudomonas*, *Pseudomonas enalia* (ATCC 14393), *Pseudomonas nigrifaciens* (ATCC 19375), and *Pseudomonas putrefaciens* (ATCC 8071), which have been reclassified respectively as *Alteromonas haloplanktis*, *Alteromonas nigrifaciens*, and *Alteromonas putrefaciens*. Similarly, e.g., *Pseudomonas acidovorans* (ATCC 15668) and *Pseudomonas testosteroni* (ATCC 11996) have since been reclassified as *Comamonas acidovorans* and *Comamonas testosteroni*, respectively; and *Pseudomonas nigrifaciens* (ATCC 19375) and *Pseudomonas piscicida* (ATCC 15057) have been reclassified respectively as *Pseudoalteromonas nigrifaciens* and *Pseudoalteromonas piscicida*. “Gram-negative Proteobacteria Subgroup 1” also includes Proteobacteria classified as belonging to any of the families: *Pseudomonadaceae*, *Azotobacteraceae* (now often called by the synonym, the “*Azotobacter* group” of *Pseudomonadaceae*), *Rhizobiaceae*, and *Methylomonadaceae* (now often called by the synonym, “*Methylococcaceae*”). Consequently, in addition to those genera otherwise described herein, further Proteobacterial genera falling within “Gram-negative Proteobacteria Subgroup 1” include: 1) *Azotobacter* group bacteria of the genus *Azorhizophilus*;

2) *Pseudomonadaceae* family bacteria of the genera *Cellvibrio*, *Oligella*, and *Teredinibacter*; 3) Rhizobiaceae family bacteria of the genera *Chelatobacter*, *Ensifer*, *Liberibacter* (also called “*Candidatus Liberibacter*”), and *Sinorhizobium*; and 4) Methylococcaceae family bacteria of the genera *Methylobacter*, *Methylocaldum*, *Methylomicrobium*, *Methylosarcina*, and *Methylosphaera*.

[0192] The host cell can be selected from “Gram-negative Proteobacteria Subgroup 16.” “Gram-negative Proteobacteria Subgroup 16” is defined as the group of Proteobacteria of the following *Pseudomonas* species (with the ATCC or other deposit numbers of exemplary strain(s) shown in parenthesis): *Pseudomonas abietaniphila* (ATCC 700689); *Pseudomonas aeruginosa* (ATCC 10145); *Pseudomonas alcaligenes* (ATCC 14909); *Pseudomonas anguilliseptica* (ATCC 33660); *Pseudomonas citronellolis* (ATCC 13674); *Pseudomonas flavescens* (ATCC 51555); *Pseudomonas mendocina* (ATCC 25411); *Pseudomonas nitroreducens* (ATCC 33634); *Pseudomonas oleovorans* (ATCC 8062); *Pseudomonas pseudoalcaligenes* (ATCC 17440); *Pseudomonas resinovorans* (ATCC 14235); *Pseudomonas straminea* (ATCC 33636); *Pseudomonas agarici* (ATCC 25941); *Pseudomonas alcaliphila*; *Pseudomonas alginovora*; *Pseudomonas andersonii*; *Pseudomonas asplenii* (ATCC 23835); *Pseudomonas azelaica* (ATCC 27162); *Pseudomonas beyerinckii* (ATCC 19372); *Pseudomonas borealis*; *Pseudomonas boreopolis* (ATCC 33662); *Pseudomonas brassicacearum*; *Pseudomonas butanovora* (ATCC 43655); *Pseudomonas cellulosa* (ATCC 55703); *Pseudomonas aurantiaca* (ATCC 33663); *Pseudomonas chlororaphis* (ATCC 9446, ATCC 13985, ATCC 17418, ATCC 17461); *Pseudomonas fragi* (ATCC 4973); *Pseudomonas hundensis* (ATCC 49968); *Pseudomonas taetrolens* (ATCC 4683); *Pseudomonas cissicola* (ATCC 33616); *Pseudomonas coronafaciens*; *Pseudomonas diterpeniphila*; *Pseudomonas elongata* (ATCC 10144); *Pseudomonas flectens* (ATCC 12775); *Pseudomonas azotoformans*; *Pseudomonas brenneri*; *Pseudomonas cedrella*; *Pseudomonas corrugata* (ATCC 29736); *Pseudomonas extremorientalis*; *Pseudomonas fluorescens* (ATCC 35858); *Pseudomonas gessardii*; *Pseudomonas libanensis*; *Pseudomonas mandelii* (ATCC 700871); *Pseudomonas marginalis* (ATCC 10844); *Pseudomonas migulae*; *Pseudomonas mucidolens* (ATCC 4685); *Pseudomonas orientalis*; *Pseudomonas rhodesiae*; *Pseudomonas synxantha* (ATCC 9890); *Pseudomonas tolaasii* (ATCC 33618); *Pseudomonas veronii* (ATCC 700474); *Pseudomonas frederiksbergensis*; *Pseudomonas geniculata* (ATCC 19374); *Pseudomonas gingeri*; *Pseudomonas graminis*; *Pseudomonas grimontii*; *Pseudomonas halodenitrificans*; *Pseudomonas halophila*; *Pseudomonas hibiscicola* (ATCC 19867); *Pseudomonas huttiensis* (ATCC 14670); *Pseudomonas hydrogenovora*; *Pseudomonas jessenii* (ATCC 700870); *Pseudomonas kilonensis*; *Pseudomonas lanceolata* (ATCC 14669); *Pseudomonas lini*; *Pseudomonas marginate* (ATCC 25417); *Pseudomonas mephitica* (ATCC

33665); *Pseudomonas denitrificans* (ATCC 19244); *Pseudomonas pertucinogena* (ATCC 190); *Pseudomonas pictorum* (ATCC 23328); *Pseudomonas psychrophila*; *Pseudomonas filva* (ATCC 31418); *Pseudomonas monteilii* (ATCC 700476); *Pseudomonas mosselii*; *Pseudomonas oryzihabitans* (ATCC 43272); *Pseudomonas plecoglossicida* (ATCC 700383); *Pseudomonas putida* (ATCC 12633); *Pseudomonas reactans*; *Pseudomonas spinosa* (ATCC 14606); *Pseudomonas balearica*; *Pseudomonas luteola* (ATCC 43273); *Pseudomonas stutzeri* (ATCC 17588); *Pseudomonas amygdali* (ATCC 33614); *Pseudomonas avellanae* (ATCC 700331); *Pseudomonas caricapapayae* (ATCC 33615); *Pseudomonas cichorii* (ATCC 10857); *Pseudomonas ficuserectae* (ATCC 35104); *Pseudomonas fuscovaginae*; *Pseudomonas meliae* (ATCC 33050); *Pseudomonas syringae* (ATCC 19310); *Pseudomonas viridiflava* (ATCC 13223); *Pseudomonas thermocarboxydovorans* (ATCC 35961); *Pseudomonas thermotolerans*; *Pseudomonas thivervalensis*; *Pseudomonas vancouverensis* (ATCC 700688); *Pseudomonas wisconsinensis*; and *Pseudomonas xiamenensis*. In one embodiment, the host cell is *Pseudomonas fluorescens*.

[0193] The host cell can also be selected from “Gram-negative Proteobacteria Subgroup 17.” “Gram-negative Proteobacteria Subgroup 17” is defined as the group of Proteobacteria known in the art as the “fluorescent *Pseudomonads*” including those belonging, e.g., to the following *Pseudomonas* species: *Pseudomonas azotoformans*; *Pseudomonas brenneri*; *Pseudomonas cedrella*; *Pseudomonas corrugata*; *Pseudomonas extremorientalis*; *Pseudomonas fluorescens*; *Pseudomonas gessardii*; *Pseudomonas libanensis*; *Pseudomonas mandelii*; *Pseudomonas marginalis*; *Pseudomonas migulae*; *Pseudomonas mucidolens*; *Pseudomonas orientalis*; *Pseudomonas rhodesiae*; *Pseudomonas synxantha*; *Pseudomonas tolaasii*; and *Pseudomonas veronii*.

[0194] Host Strain Backgrounds

[0195] Host cells, strains and expression constructs useful in practicing the methods of the invention can be identified or made using reagents and methods known to those of skill in the art and described in the literature. For example, U.S. Pat. No. 8,288,127, “Protein Expression Systems,” incorporated herein by reference in its entirety, describes production of a recombinant polypeptide by introduction of a nucleic acid construct into an auxotrophic *Pseudomonas fluorescens* host cell comprising a chromosomal lacI gene insert (e.g., *lsc::lacI^{Q1}*). The nucleic acid construct comprises a nucleotide sequence encoding the recombinant polypeptide operably linked to a promoter capable of directing expression of the nucleic acid in the host cell, and also comprises a nucleotide sequence encoding an auxotrophic selection marker. The auxotrophic selection marker is a polypeptide that restores prototrophy to the auxotrophic host cell. In embodiments, the cell is auxotrophic for proline, uracil, or combinations thereof. In

embodiments, the host cell is derived from MB101 (ATCC deposit PTA-7841). U.S. Pat. No. 8,288,127, "Protein Expression Systems," and Schneider, et al., 2005, "Auxotrophic markers *pyrF* and *proC* can replace antibiotic markers on protein production plasmids in high-cell-density *Pseudomonas fluorescens* fermentation," Biotechnol. Progress 21(2): 343-8, both incorporated herein by reference in their entirety, describe a production host strain auxotrophic for uracil that was constructed by deleting the *pyrF* gene in strain MB101. The *pyrF* gene was cloned from strain MB214 (ATCC deposit PTA-7840) to generate a plasmid that complements the *pyrF* deletion to restore prototrophy. In particular embodiments, a dual *pyrF-proC* dual auxotrophic selection marker system in a *P. fluorescens* host cell is used. Given the published literature, a *pyrF* deleted production host strain as described can be produced by one of skill in the art using known methods and used as the background for introducing other desired genomic changes, including those described herein as useful in practicing the methods of the invention. It would be understood by one of skill in the art that a production host strain useful in the methods of the present invention can be generated using a publicly available host cell, for example, *P. fluorescens* MB101, e.g., by inactivating the genes encoding PyrF, Prc1, Prc2, MepM1, and optionally gene encoding an additional protease or autolytic factor, using any of many suitable methods known in the art and described in the literature. It is also understood that a prototrophy restoring plasmid can be transformed into the strain, e.g., a plasmid carrying the *pyrF* gene from strain MB214, using any suitable method known in the art and described in the literature. Additionally, in such strains inactivated protease and folding modulator overexpression constructs may be introduced, using methods well known in the art.

[0196] In embodiments, a *P. fluorescens* host strain used in the methods of the invention is DC1032 ($\Delta prc1$, $\Delta prc2$, $\Delta hslUV$, $\Delta pyrF$, $lsc::lacI^{Q1}$), a derivative of deposited strain MB101 in which the genes *pyrF* is deleted, and the *E. coli* *lacI* transcriptional repressor is inserted and fused with the levansucrase gene (*lsc*). Sequences for these genes and methods for their use are known in the art and described in the literature, e.g., in U.S. Pat. Nos. 8,288,127, 8,017,355, "Mannitol induced promoter systems in bacterial host cells," and 7,794,972, "Benzoate-and anthranilate-inducible promoters," each incorporated by reference herein.

[0197] A *Pseudomonas* host cell equivalent to a host cell as listed in **Table 4**, e.g., DC1032, DC954, or DC454, or any of the host cells or expression strains described herein can be constructed from MB101 using methods described herein and in the published literature. In embodiments, a host cell equivalent to DC1032 or DC954 is used. Host cell DC454 is described by Schneider, et al., 2005, where it is referred to as DC206, and in U.S. Pat. No. 8,569,015, "rPA Optimization," incorporated herein by reference in its entirety. DC206 is the same strain as

DC454; it was renamed DC454 after passage three times in animal-free media. DC454 is parental to DC1032 and DC954.

[0198] One of ordinary skill in the art will appreciate that in embodiments, a host cell genomic deletion or mutation (e.g., an inactivating or debilitating mutation) can be made by, e.g., allele exchange, using a deletion plasmid carrying regions that flank the gene to be deleted, which does not replicate in *P. fluorescens*. The deletion plasmid can be constructed by PCR amplifying the gene to be deleted, including the upstream and downstream regions of the gene to be deleted. The deletion can be verified by sequencing a PCR product amplified from genomic DNA using analytical primers, observed after separation by electrophoresis in an agarose slab gel, followed by DNA sequencing of the fragment. In embodiments, a gene is inactivated by complete deletion, partial deletion, or mutation, e.g., frameshift, point, or insertion mutation.

[0199] In embodiments, a strain used in the context of the present invention has been transformed with an FMO plasmid according to methods known in the art. The genotypes for certain examples of recombinant protein expression strains and corresponding host cells useful for expressing recombinant proteins according to the methods of the invention are set forth in **Table 10**. In embodiments, a host cell equivalent to any host cell described in **Table 4** is transformed with an expression vector as described herein, to obtain an expression strain equivalent to one described herein for expressing a recombinant protein of interest using the methods of the invention. As described, appropriate expression strains can be similarly derived according to methods set forth herein and in the literature.

[0200] Expression Systems

[0201] An appropriate bacterial expression system useful for producing the recombinant protein of interest according to the present methods can be identified by one of skill in the art based on the teachings herein. In some embodiments, an expression construct comprising a nucleotide sequence encoding a recombinant protein of interest is provided as part of an inducible expression vector. In embodiments, a host cell that has been transformed with the expression vector is cultured, and expression of the recombinant protein of interest from the expression vector is induced. The expression vector can be, for example, a plasmid. In embodiments, the expression vector is a plasmid encoding a recombinant protein coding sequence further comprising a selection marker, and the host cells are grown under selective conditions that allow maintenance of the plasmid. In embodiments, the expression construct is integrated into the host cell genome. In embodiments, the expression construct encodes a recombinant protein of interest fused to a secretory signal that can direct the recombinant protein of interest to the periplasm.

[0202] Methods for expressing heterologous proteins, including useful regulatory sequences (e.g., promoters, secretion signals, and ribosome binding sites), in host cells useful in the methods of the present invention, are described in the literature, e.g., in U.S. Patent No. 7,618,799, “Bacterial leader sequences for increased expression,” in U.S. Pat. No. 7,985,564, “Expression systems with Sec-system secretion,” in U.S. Pat. Nos. 9,394,571 and 9,580,719, U.S. Pat. No. 9,458,487 and U.S. Pat. No. 9,453,251, U.S. Pat. No. 8,603,824, U.S. Pat. No. 8,530,171, “High level expression of recombinant toxin proteins,” U.S. Pat. No. 10,118,956, U.S. Pat. No. 5,888,808, Bacterial polypeptide expression employing tryptophan promoter-operator,” U.S. Pat. No. 9,534,217, “Method of creating a library of bacterial clones with varying levels of gene expression,” and Vellanoweth, R.L., and Rabinowitz, J. C., May 1992, “The influence of ribosome-binding-site elements on translational efficiency in *Bacillus subtilis* and *Escherichia coli* in vivo,” Molecular Microbiology 6(9):1105-1114, each incorporated herein by reference in its entirety. In embodiments, a secretion leader used in the context of the present invention is a secretion leader as disclosed in any of U.S. Pat. Nos. 7,618,799, 7,985,564, 9,394,571, 9,580,719, 9,453,251, 8,603,824, 8,530,171, and 10,118,956. These patents also describe bacterial host strains useful in practicing the methods herein, that have been engineered to overexpress folding modulators or wherein protease mutations have been introduced, in order to increase heterologous protein expression.

[0203] Promoters used in accordance with the present invention may be constitutive promoters or regulated promoters. Examples of inducible promoters include those of the family derived from the *lac* promoter (i.e. the *lacZ* promoter), e.g., the *tac* and *trc* promoters described in U.S. Pat. No. 4,551,433, “Microbial Hybrid Promoters,” incorporated herein by reference, as well as Ptac16, Ptac17, PtacII, PlacUV5, and the T7lac promoter. In embodiments, the promoter is not derived from the host cell organism. In embodiments, the promoter is derived from an *E. coli* organism. In embodiments, a *lac* promoter is used to regulate expression of a recombinant protein of interest from a plasmid. In the case of the *lac* promoter derivatives or family members, e.g., the *tac* promoter, an inducer is IPTG (isopropyl- β -D-1-thiogalactopyranoside, “isopropylthiogalactoside”). In embodiments, IPTG is added to the host cell culture to induce expression of the recombinant protein of interest from a *lac* promoter in a *Pseudomonas* host cell according to methods known in the art and described in the literature, e.g., in U.S. Pat. No. 9,458,487 and U.S. Pat. No. 9,453,251.

[0204] Examples of non-*lac* promoters useful in expression systems according to the present invention include, P_R (induced by high temperature), P_L (induced by high temperature), P_m (induced by Alkyl- or halo-benzoates), P_u (induced by alkyl- or halo-toluenes), or P_{sal} (induced by salicylates), described in, e.g. J. Sanchez-Romero & V. De Lorenzo (1999) Manual of Industrial

Microbiology and Biotechnology (A. Demain & J. Davies, eds.) pp. 460-74 (ASM Press, Washington, D.C.); H. Schweizer (2001) *Current Opinion in Biotechnology*, 12:439-445; and R. Slater & R. Williams (2000) *Molecular Biology and Biotechnology* (J. Walker & R. Rapley, eds.) pp. 125-54 (The Royal Society of Chemistry, Cambridge, UK). A promoter having the nucleotide sequence of a promoter native to the selected bacterial host cell also may be used to control expression of the expression construct encoding the polypeptide of interest, e.g., a *Pseudomonas* anthranilate or benzoate operon promoter (Pant, Pben). Tandem promoters may also be used in which more than one promoter is covalently attached to another, whether the same or different in sequence, e.g., a Pant-Pben tandem promoter (interpromoter hybrid) or a Plac-Plac tandem promoter, derived from the same or different organisms. In embodiments, the promoter is Pmtl, as described in, e.g., U.S. Pat. Nos. 7,476,532, and 8,017,355, both titled "Mannitol induced promoter systems in bacterial host cells," incorporated by reference herein in their entirety.

[0205] Regulated (inducible) promoters utilize promoter regulatory proteins in order to control transcription of the gene of which the promoter is a part. Where a regulated promoter is used herein, a corresponding promoter regulatory protein will also be part of an expression system according to the present invention. Examples of promoter regulatory proteins include: activator proteins, e.g., *E. coli* catabolite activator protein, MalT protein; AraC family transcriptional activators; repressor proteins, e.g., *E. coli* Lad proteins; and dual-function regulatory proteins, e.g., *E. coli* NagC protein. Many regulated-promoter/promoter-regulatory-protein pairs are known in the art. In some embodiments, a promoter used to transcribe a gene encoding a recombinant protein of interest produced using the present compositions and methods is selected from: a tac promoter, a mannitol promoter, a Pben, a T7 promoter, a lac promoter, a T5 promoter, a xylose promoter, a Trp promoter, and an arabinose promoter. When more than one expression construct is used to produce the recombinant protein of interest, more than one different promoter may be used.

[0206] Promoter regulatory proteins interact with an effector compound, i.e., a compound that reversibly or irreversibly associates with the regulatory protein so as to enable the protein to either release or bind to at least one DNA transcription regulatory region of the gene that is under the control of the promoter, thereby permitting or blocking the action of a transcriptase enzyme in initiating transcription of the gene. Effector compounds are classified as either inducers or co-repressors, and these compounds include native effector compounds and gratuitous inducer compounds. Many regulated-promoter/promoter-regulatory-protein/effector-compound trios are known in the art. Although an effector compound can be used throughout the cell culture or fermentation, in a preferred embodiment in which a regulated promoter is used, after growth of a

desired quantity or density of host cell biomass, an appropriate effector compound is added to the culture to directly or indirectly result in expression of the desired gene(s) encoding the recombinant protein of interest.

[0207] In embodiments wherein a lac family promoter is utilized, a *lacI* gene can also be present in the system. The *lacI* gene, which is normally a constitutively expressed gene, encodes the Lac repressor protein LacI protein, which binds to the lac operator of *lac* family promoters. Thus, where a *lac* family promoter is utilized, the *lac* gene can also be included and expressed in the expression system.

[0208] Expression Vectors

[0209] At least one nucleic acid sequence encoding a recombinant protein of interest can be introduced into a suitable expression vector(s) to produce either the recombinant protein of interest, an overexpressed protein, e.g., a chaperone, folding modulator, or inactivated protease as described herein, or both the recombinant protein of interest, and the overexpressed protein may be produced using the same expression vector. The expression vector can be a plasmid. An expression vector may be selected for use in the context of the present invention by one of skill in the art as desired and appropriate, from commercially available expression vectors. In some embodiments, a plasmid encoding a recombinant protein of interest can comprise a selection marker, and host cells maintaining the plasmid can be grown under selective conditions. In some embodiments, the plasmid does not comprise a selection marker. In some embodiments, the expression vector is integrated into the host cell genome. In some embodiments, the expression vector encodes a recombinant protein of interest fused to a secretion signal that can direct the expressed recombinant protein of interest to the periplasm. In some embodiments, the expression vector encodes a recombinant protein of interest fused to a secretion signal that can direct the expressed recombinant protein of interest to the cytoplasm. In some embodiments, an expression vector encodes a Fab', e.g., an anti-TNF-alpha Fab', fused to a periplasmic secretion signal that can direct the expressed Fab' to the periplasm.

[0210] Recombinant proteins of interest that can be produced using the present compositions and methods are described herein. Amino acid sequences of recombinant proteins of interest, and potential coding sequences, may readily be obtained by those of skill in the art. The amino acid sequences of the heavy and light chains of an anti-TNF-alpha Fab', and examples of nucleotide sequences encoding the Fab', are provided in **Table 14**, the **Table of Sequences**, herein.

Other Regulatory Elements

[0211] In some embodiments, other regulatory elements are present in the expression construct encoding the recombinant protein of interest. In embodiments, the soluble recombinant protein

of interest is present in either the cytoplasm or periplasm of the cell during production. Secretion leaders useful for targeting a recombinant protein of interest to either compartment are described herein. In embodiments, an expression construct of the present invention encodes a recombinant protein of interest fused to a secretion signal that can transport the recombinant protein of interest to the cytoplasm of a *Pseudomonad* cell. In embodiments, an expression construct encodes a recombinant protein of interest fused to a secretion leader that can transport a recombinant protein of interest to the periplasm of a *Pseudomonad* cell. In embodiments, the secretion leader is cleaved from the recombinant protein of interest.

[0212] Other elements include, but are not limited to, transcriptional enhancer sequences, translational enhancer sequences, other promoters, activators, translational start and stop signals, transcription terminators, cistronic regulators, polycistronic regulators, tag sequences, such as nucleotide sequence tags and tag polypeptide coding sequences, which facilitate identification, separation, purification, and/or isolation of an expressed polypeptide, as previously described. In some embodiments, the expression construct includes, in addition to the protein coding sequence, any of the following regulatory elements operably linked thereto: a promoter, a ribosome binding site (RBS), a transcription terminator, and translational start and stop signals. Useful RBSs can be obtained from any of the species useful as host cells in expression systems according to, e.g., U.S. Pat. No. 10,118,956 and U.S. 9,580,719, previously referenced. Many RBSs are known, e.g., those described in and referenced by D. Frishman et al., *Gene* 234(2):257-65 (8 Jul. 1999); and B. E. Suzek et al., *Bioinformatics* 17(12):1123-30 (December 2001), incorporated herein by reference. In addition, either native or synthetic RBSs may be used, e.g., those described in: EP 0207459 (synthetic RBSs); O. Ikehata et al., *Eur. J. Biochem.* 181(3):563-70 (1989). In some embodiments, a “Hi” ribosome binding site, aggagg, (SEQ ID NO: 59) is used in the construct. Ribosome binding sites, including the optimization of spacing between the RBS and translation initiation codon, are described in the literature, e.g., by Chen, et al., 1994, “Determination of the optimal aligned spacing between the Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs,” *Nucleic Acids Research* 22(23):4953-4957, and Ma, et al., 2002, “Correlations between Shine-Dalgarno Sequences and Gene Features Such as Predicted Expression Levels and Operon Structures,” *J. Bact.* 184(20): 5733-45, incorporated herein by reference.

[0213] Further examples of methods, vectors, and translation and transcription elements, and other elements useful in the present invention are well known in the art and described in, e.g.: U.S. Pat. No. 5,055,294 to Gilroy and U.S. Pat. No. 5,128,130 to Gilroy et al.; U.S. Pat. No. 5,281,532 to Rammler et al.; U.S. Pat. Nos. 4,695,455 and 4,861,595 to Barnes et al.; U.S. Pat.

No. 4,755,465 to Gray et al.; and U.S. Pat. No. 5,169,760 to Wilcox, all incorporated herein by reference, as well as in other publications incorporated herein by reference.

Secretion Leader Sequences

[0214] In embodiments, a secretion signal or leader coding sequence is fused to the N-terminus of the sequence encoding the recombinant protein of interest. Use of secretion signal sequences can increase production of recombinant proteins in bacteria. Additionally, many types of proteins require secondary modifications that are inefficiently achieved using known methods. Secretion leader utilization can increase the harvest of properly folded proteins by secreting the protein from the intracellular environment. In gram-negative bacteria, a protein secreted from the cytoplasm can end up in the periplasmic space, attached to the outer membrane, or in the extracellular broth. These methods may avoid formation of inclusion bodies. Secretion of proteins into the periplasmic space also has the effect of facilitating proper disulfide bond formation (Bardwell et al., 1994, *Phosphate Microorg*, Chapter 45, 270-5, and Manoil, 2000, *Methods in Enzymol.* 326:35-47). Other benefits of secretion of recombinant protein include more efficient isolation of the protein, proper folding and disulfide bond formation of the protein leading to an increase in yield represented by, e.g., the percentage of the protein in active form, reduced formation of inclusion bodies and reduced toxicity to the host cell, and an increased percentage of the recombinant protein in soluble form. The potential for excretion of the protein of interest into the culture medium can also potentially promote continuous, rather than batch, culture for protein production. Secretion signals are described, e.g., in U.S. Patent No. 7,618,799, U.S. Pat. No. 7,985,564, and U.S. Pat. App. Pub. No. 2019/0127744, "Bacterial leader sequences for periplasmic protein expression," each incorporated herein by reference in its entirety, as well as by U.S. Pat. No. 10,118,956.

[0215] In some embodiments, the recombinant protein of interest is targeted to the periplasm of the host cell or into the extracellular space. In some embodiments, the expression vector further comprises a nucleotide sequence encoding a secretion signal polypeptide operably linked to the nucleotide sequence encoding the recombinant protein of interest.

[0216] Codon Optimization

[0217] The present invention contemplates the use of any appropriate coding sequence for the recombinant protein of interest, including any sequence that has been optimized for expression in the host cell being used. A nucleic acid sequence encoding the recombinant protein of interest may be codon-optimized to improve expression in the recombinant gram-negative bacterial host cell, as understood by one of skill in the art. For example, optimization of codons for expression in a *Pseudomonas* host strain is described, e.g., in U.S. Pat. App. Pub. No. 2007/0292918, "Codon Optimization Method," incorporated herein by reference in its entirety. Codon

optimization for expression in *E. coli* is described, e.g., by Welch, et al., 2009, PLoS One, “Design Parameters to Control Synthetic Gene Expression in *Escherichia coli*, 4(9): e7002, incorporated by reference herein. It is understood that any suitable sequence encoding a recombinant protein of interest can be generated as desired according to methods well known by those of skill in the art.

[0218] Expression Constructs

[0219] An appropriate expression construct for producing a recombinant protein of interest according to the methods of the invention may be selected by one of skill in the art in view of the present disclosure.

[0220] In some embodiments, a recombinant protein of interest produced in a recombinant gram-negative host cell of the present invention is encoded by an expression vector comprising at least one expression construct encoding the recombinant protein of interest, wherein the expression construct comprises at least one nucleic acid sequence encoding the recombinant protein of interest. In some embodiments, at least two nucleic acid sequences encoding the recombinant protein of interest are transcribed from the same promoter (co-transcribed). In some embodiments at least two nucleic acid sequences encoding the recombinant protein of interest are transcribed from different promoters (not co-transcribed). When not co-transcribed, each of the least two nucleic acid sequences encoding the at least two nucleic acid sequences encoding the recombinant protein of interest may be produced from the same expression vector or separate expression vectors. In some embodiments, a nucleic acid sequence encoding a recombinant protein of interest is operably linked to a nucleic acid sequence encoding a secretion signal. In some embodiments, each of at least two nucleic acid sequences encoding a recombinant protein of interest is individually operably linked to a nucleic acid sequence encoding the same or different secretion signal. In some embodiments, each nucleic acid sequence encoding a recombinant protein of interest in a host cell is individually operably linked to a nucleic acid sequence independently selected from the periplasmic secretion signals having the amino acid sequence set forth as: SEQ ID NO: 11, 13, 25, or 26.

[0221] In some embodiments, a recombinant gram-negative bacterial host cell of the present invention is transformed with expression vector(s) comprising the at least one expression construct encoding the recombinant protein of interest. In some embodiments, the transformed recombinant gram-negative bacterial host cell is deficient in a tail-specific protease activity, and a Mep1 endopeptidase activity. In some embodiments, the transformed recombinant gram-negative bacterial host cell is further: optionally deficient in at least one additional protease activity, optionally deficient in at least one autolytic factor activity, optionally overexpresses one or more inactivated protease, optionally overexpresses one or more chaperone or folding

modulator, optionally has a functional MepS1 protease, and optionally has a functional MepS2 protease, each as described elsewhere herein in detail. In some embodiments, the transformed recombinant gram-negative bacterial host cell is selected from: a *Pseudomonad* host cell; an *E. coli* host cell; an *Erwinia* host cell, a *Salmonella* host cell, a *Shigella* host cell, a *Moraxella* host cell, a *Helicobacter* host cell, a *Legionella* host cell, a *Neisseria* host cell, a *Haemophilus* host cell, a *Acinetobacter* host cell, a *Bacteroides* host cell, a *Xylella* host cell, a *Citrobacter* host cell, an *Enterobacter* host cell, a *Klebsiella* host cell, a *Yersinia* host cell, a *Serratia* host cell, a *Proteus* host cell, and a *Vibrio* host cell. In some embodiments, the *Pseudomonad* host cell is a *Pseudomonas* host cell. In some embodiments, the *Pseudomonas* host cell is *P. fluorescens*, *P. putida*, or *P. aeruginosa*.

[0222] In some embodiments, the recombinant gram-negative bacterial host cell transformed with expression vector(s) comprising the at least one expression construct encoding the recombinant protein of interest is: (i) *lsc::lacIQ1*; (ii) *Prcl* deficient; (ii) *Prcl* deficient; (iii) *HslU* deficient; (iv) *HslV* deficient; (v) *MepM1* deficient; and (vi) *PyrF* deficient; wherein the host cell is optionally deficient in a serralysin precursor that is: a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; optionally overexpresses a *DsbC*; optionally overexpresses an inactivated *DegP2*; and optionally overexpresses a *PDIA6*.

[0223] In some embodiments, a recombinant protein or polypeptide of interest is produced in a recombinant gram-negative bacterial host cell that is any one of strains STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the recombinant protein or polypeptide of interest is produced in a recombinant gram-negative bacterial host cell that has the genotype (genomic modifications) of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, any one of strains STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the recombinant protein or polypeptide of interest is produced in a recombinant gram-negative bacterial host cell that has the genotype of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, STR94975, STR94976, or STR94977.

[0224] In some embodiments, an antibody to be produced in a recombinant gram-negative host cell of the present invention is encoded by an expression vector comprising at least one expression construct encoding the antibody, wherein the expression construct comprises at least two nucleic acid sequences encoding a heavy chain and at least two nucleic acid sequences

encoding a light chain. In some embodiments two or more of the at least two nucleic acid sequences encoding the heavy chain and the at least two nucleic acid sequence encoding the light chain are transcribed from the same promoter (co-transcribed). In some embodiments the at least two nucleic acid sequences encoding a heavy chain and the at least two nucleic acid sequences encoding the light chain are transcribed from different promoters (not co-transcribed). When not co-transcribed, each of the at least two nucleic acid sequence encoding the antibody heavy chain and the at least two nucleic acid sequences encoding the antibody light chain may be produced from the same expression vector or multiple expression vectors. In some embodiments, each heavy chain encoding nucleic acid sequence and each light chain encoding nucleic acid sequence is individually operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, resulting in a secretion signal-heavy chain fusion and a secretion signal-light chain fusion. In some embodiments, each heavy chain encoding nucleic acid sequence is operably linked to a nucleic acid sequence encoding a periplasmic secretion signal having the amino acid sequence set forth as SEQ ID NO: 11. In some embodiments, each light chain encoding nucleic acid sequence is operably linked to a nucleic acid sequence encoding a periplasmic secretion signal having the amino acid sequence set forth as SEQ ID NO: 13.

[0225] In some embodiments, a recombinant gram-negative bacterial host cell of the present invention is transformed with expression vector(s) comprising the at least one expression construct encoding the antibody. In some embodiments, the transformed recombinant gram-negative bacterial host cell is deficient in a tail-specific protease activity, and a Mep1 endopeptidase activity. In some embodiments, the transformed recombinant gram-negative bacterial host cell is further: optionally deficient in at least one additional protease activity, optionally deficient in at least one autolytic factor activity, optionally overexpresses one or more inactivated protease, optionally overexpresses one or more chaperone or folding modulator, optionally has a functional MepS1 protease, and optionally has a functional MepS2 protease, each as described elsewhere herein in detail. In some embodiments, the transformed recombinant gram-negative bacterial host cell is selected from: a *Pseudomonad* host cell; an *E. coli* host cell; an *Erwinia* host cell, a *Salmonella* host cell, a *Shigella* host cell, a *Moraxella* host cell, a *Helicobacter* host cell, a *Legionella* host cell, a *Neisseria* host cell, a *Haemophilus* host cell, a *Acinetobacter* host cell, a *Bacteroides* host cell, a *Xylella* host cell, a *Citrobacter* host cell, an *Enterobacter* host cell, a *Klebsiella* host cell, a *Yersinia* host cell, a *Serratia* host cell, a *Proteus* host cell, and a *Vibrio* host cell. In some embodiments, the *Pseudomonad* host cell is a *Pseudomonas* host cell. In some embodiments, the *Pseudomonas* host cell is *P. fluorescens*, *P. putida*, or *P. aeruginosa*. In some embodiments, the transformed recombinant gram-negative bacterial host cell is not an *E. coli* host cell.

[0226] In some embodiments, the recombinant gram-negative bacterial host cell transformed with expression vector(s) comprising the at least one expression construct encoding the antibody is: (i) *lsc::lacIQ1*; (ii) *Prc1* deficient; (ii) *Prc2* deficient; (iii) *HslU* deficient; (iv) *HslV* deficient; (v) *MepM1* deficient; (vi) *PyrF* deficient; wherein the host cell is optionally deficient in a serralyisin precursor that is: a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; optionally overexpresses a *DsbC*; optionally overexpresses an inactivated *DegP2*; and optionally overexpresses a *PDIA6*.

[0227] In some embodiments, an antibody is produced in a recombinant gram-negative bacterial host cell that is any one of strains STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the antibody is produced in a recombinant gram-negative bacterial host cell that has the genotype of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, any one of strains STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the antibody is produced in a recombinant gram-negative bacterial host cell that has the genotype of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, STR94975, STR94976, and STR94977.

[0228] In some embodiments, a Fab or Fab', e.g., a human TNF-alpha Fab', to be produced in a recombinant gram-negative host cell of the present invention is encoded by an expression vector comprising at least one expression construct encoding the Fab or Fab', wherein the expression construct comprises at least one nucleic acid sequence encoding a heavy chain and at least one nucleic acid sequence encoding a light chain. In some embodiments, the heavy chain has the amino acid sequence set forth as SEQ ID NO: 21, and the light chain has the amino acid sequence set forth as SEQ ID NO: 23. In some embodiments the at least one nucleic acid sequence encoding a heavy chain and the at least one nucleic acid sequence encoding a light chain are transcribed from the same promoter (co-transcribed). In some embodiments the at least one nucleic acid sequence encoding a heavy chain and the at least one nucleic acid sequence encoding a light chain are transcribed from different promoters (not co-transcribed). When not co-transcribed, the at least one nucleic acid sequence encoding the Fab or Fab' heavy chain and the at least one nucleic acid sequence encoding the Fab or Fab' light chain may be produced from the same expression vector or separate expression vectors. In some embodiments, each heavy chain encoding nucleic acid sequence and each light chain encoding nucleic acid sequence is individually operably linked to a nucleic acid sequence encoding a periplasmic secretion signal,

resulting in a secretion signal-heavy chain fusion and a secretion signal-light chain fusion. In some embodiments, each Fab or Fab' heavy chain encoding nucleic acid sequence is operably linked to a nucleic acid sequence encoding a periplasmic secretion signal having the amino acid sequence set forth as SEQ ID NO: 11. In some embodiments, each Fab or Fb' light chain encoding nucleic acid sequence is operably linked to a nucleic acid sequence encoding a periplasmic secretion signal having the amino acid sequence set forth as SEQ ID NO: 13. In some embodiments, a human TNF-alpha Fab' secretion signal-heavy chain fusion has the amino acid sequence set forth as SEQ ID NO: 25, and a human TNF-alpha Fab' secretion signal-light chain fusion has the amino acid sequence set forth as SEQ ID NO: 26.

[0229] In some embodiments, the recombinant protein or polypeptide of interest is produced in a recombinant gram-negative bacterial host cell that has the following genotype: Δ prc1, Δ prc2, Δ hslU, Δ hslV, Δ mepM1, Δ RXF04495.2, Δ pyrF, and $\text{lsc}::\text{lacI}^{\text{Q1}}$. In some embodiments, the recombinant protein or polypeptide of interest is an anti-TNF-alpha Fab', and the host cell further comprises a plasmid comprising expression constructs selected from:

a) a nucleic acid sequence encoding an anti-TNF-alpha Fab' heavy chain (HC) having the amino acid sequence set forth as SEQ ID NO: 21, a nucleic acid sequence encoding an anti-TNF-alpha Fab' light chain (LC) having the amino acid sequence set forth as SEQ ID NO: 23, wherein the anti-TNF-alpha Fab' HC and the anti-TNF-alpha Fab' LC are co-transcribed, and a nucleic acid sequence encoding PyrF;

b) a nucleic acid sequence encoding an anti-TNF-alpha Fab' HC having the amino acid sequence set forth as SEQ ID NO: 21, a nucleic acid sequence encoding an anti-TNF-alpha Fab' LC having the amino acid sequence set forth as SEQ ID NO: 23, wherein the anti-TNF-alpha Fab' HC and the anti-TNF-alpha Fab' LC are co-transcribed, a nucleic acid sequence encoding DegP2 S219A having the amino acid sequence set forth as SEQ ID NO: 29, and a nucleic acid sequence encoding PyrF;

c) a nucleic acid sequence encoding an anti-TNF-alpha Fab' HC having the amino acid sequence set forth as SEQ ID NO: 21, a nucleic acid sequence encoding an anti-TNF-alpha Fab' LC having the amino acid sequence set forth as SEQ ID NO: 23, and a nucleic acid sequence encoding DegP2 S219A having the amino acid sequence set forth as SEQ ID NO: 29, wherein the anti-TNF-alpha Fab' HC, the anti-TNF-alpha Fab' LC, and DegP2 S219A are co-transcribed, and a nucleic acid sequence encoding PyrF; and

d) a nucleic acid sequence encoding an anti-TNF-alpha Fab' HC having the amino acid sequence set forth as SEQ ID NO: 21, a nucleic acid sequence encoding an anti-TNF-alpha Fab' LC having the amino acid sequence set forth as SEQ ID NO: 23, and a nucleic acid sequence encoding PDIA6 having the amino acid sequence set forth as SEQ ID NO: 27, wherein the anti-

TNF-alpha Fab' HC, the anti-TNF-alpha Fab' LC, and PDIA6 are co-transcribed, and a nucleic acid sequence encoding PyrF.

[0230] In some embodiments, the recombinant protein or polypeptide of interest is an anti-TNF-alpha Fab', and the host cell further comprises a plasmid comprising expression constructs selected from:

a) a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' heavy chain (HC) fusion having the amino acid sequence set forth as SEQ ID NO: 25, a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' light chain (LC) fusion having the amino acid sequence set forth as SEQ ID NO: 26, wherein the secretion leader-anti-TNF-alpha Fab' HC fusion and the secretion leader-anti-TNF-alpha Fab' LC fusion are co-transcribed, and a nucleic acid sequence encoding PyrF;

b) a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' HC fusion having the amino acid sequence set forth as SEQ ID NO: 25, a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' LC fusion having the amino acid sequence set forth as SEQ ID NO: 26, wherein the secretion leader-anti-TNF-alpha Fab' HC fusion and the secretion leader-anti-TNF-alpha Fab' LC fusion are co-transcribed, a nucleic acid sequence encoding DegP2 S219A having the amino acid sequence set forth as SEQ ID NO: 29, and a nucleic acid sequence encoding PyrF;

c) a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' HC fusion having the amino acid sequence set forth as SEQ ID NO: 25, a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' LC fusion having the amino acid sequence set forth as SEQ ID NO: 26, and a nucleic acid sequence encoding DegP2 S219A having the amino acid sequence set forth as SEQ ID NO: 29, wherein the secretion leader-anti-TNF-alpha Fab' HC fusion, the secretion leader-anti-TNF-alpha Fab' LC fusion, and DegP2 S219A are co-transcribed, and a nucleic acid sequence encoding PyrF; and

d) a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' HC fusion having the amino acid sequence set forth as SEQ ID NO: 25, a nucleic acid sequence encoding a secretion leader-anti-TNF-alpha Fab' LC fusion having the amino acid sequence set forth as SEQ ID NO: 26, and a nucleic acid sequence encoding PDIA6 having the amino acid sequence set forth as SEQ ID NO: 27, wherein the secretion leader-anti-TNF-alpha Fab' HC fusion, the secretion leader-anti-TNF-alpha Fab' LC fusion, and PDIA6 are co-transcribed, and a nucleic acid sequence encoding PyrF.

[0231] In some embodiments, a recombinant gram-negative bacterial host cell of the present invention is transformed with expression vector(s) comprising the at least one expression construct encoding the Fab or Fab'. In some embodiments, the transformed recombinant gram-

negative bacterial host cell is deficient in a tail-specific protease activity, and a Mep1 endopeptidase activity. In some embodiments, the transformed recombinant gram-negative bacterial host cell is further: optionally deficient in at least one additional protease activity, optionally deficient in at least one autolytic factor activity, optionally overexpresses one or more inactivated protease, optionally overexpresses one or more chaperone or folding modulator, optionally has a functional MepS1 protease, and optionally has a functional MepS2 protease, each as described elsewhere herein in detail. In some embodiments, the transformed recombinant gram-negative bacterial host cell is selected from: a *Pseudomonad* host cell; an *E. coli* host cell; an *Erwinia* host cell, a *Salmonella* host cell, a *Shigella* host cell, a *Moraxella* host cell, a *Helicobacter* host cell, a *Legionella* host cell, a *Neisseria* host cell, a *Haemophilus* host cell, a *Acinetobacter* host cell, a *Bacteroides* host cell, a *Xylella* host cell, a *Citrobacter* host cell, an *Enterobacter* host cell, a *Klebsiella* host cell, a *Yersinia* host cell, a *Serratia* host cell, a *Proteus* host cell, and a *Vibrio* host cell. In some embodiments, the *Pseudomonad* host cell is a *Pseudomonas* host cell. In some embodiments, the *Pseudomonas* host cell is *P. fluorescens*, *P. putida*, or *P. aeruginosa*. In some embodiments, the transformed recombinant gram-negative bacterial host cell is not an *E. coli* host cell.

[0232] In some embodiments, the recombinant gram-negative bacterial host cell transformed with expression vector(s) comprising the at least one expression construct encoding the Fab or Fab', e.g., an anti-TNF Fab', is: (i) *lsc::lacIQ1*; (ii) *Prc1* deficient; (ii) *Prc2* deficient; (iii) *HslU* deficient; (iv) *HslV* deficient; (v) *MepM1* deficient; (vi) *PyrF* deficient; wherein the host cell is optionally deficient in a serralyisin precursor that is: a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; optionally overexpresses a *DsbC*; optionally overexpresses an inactivated *DegP2*; and optionally overexpresses a *PDIA6*.

[0233] In some embodiments, the recombinant gram-negative bacterial host cell is any one of strains STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the recombinant gram-negative bacterial host cell that the genotype (genomic modifications) of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, any one of strains STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the recombinant gram-negative bacterial host cell that has the genotype of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, STR94975, STR94976, or STR94977.

[0234] In some embodiments, the recombinant gram-negative bacterial host cell has a genotype of, has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, and comprises the at least one expression construct encoding an anti-TNF Fab' of expression strain STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, or STR94977. In some embodiments, the recombinant gram-negative bacterial host cell has a genotype of, has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, and comprises the at least one expression construct encoding an anti-TNF Fab' of expression strain STR94975, STR94976, or STR94977.

[0235] In some embodiments, a recombinant protein of interest, an antibody, a Fab or Fab', or an anti-TNF Fab' is produced according to the methods described herein, in a recombinant gram-negative bacterial host cell that has a genotype of, and/or has the protease deficiency, inactivated protease, and folding modulator overexpression profile of, expression strain STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, or STR94977. In some embodiments, an anti-TNF Fab' is produced according to methods described herein, in a recombinant gram-negative bacterial host cell that has a genotype of, and has the protease deficiency, inactivated protease, and folding modulator overexpression profile of expression strain STR94975, STR94976, or STR94977, and comprises the at least one expression construct encoding the anti-TNF Fab', e.g., an anti-TNF Fab' having the heavy chain sequence as set forth in SEQ ID NO: 25 and the light chain sequence as set forth in SEQ ID NO: 26. In some embodiments, the Fab' expression construct is that of an expression strain selected from: STR92557, STR87639, STR92567, STR94974, STR94975, STR94976, and STR94977. In some embodiments, the anti-TNF Fab' produced from a host strain of the present invention is produced in soluble, active, and/or intact form at a titer of about 0.2 to about 5 g/L.

[0236] Fermentation Format

[0237] A recombinant protein of interest may be produced using the methods as described herein, by culturing the recombinant gram-negative bacterial host cells transformed with a plasmid encoding the recombinant protein of interest (an expression strain) under suitable fermentation conditions. Any fermentation format, e.g., a batch, fed-batch, semi-continuous, or continuous fermentation mode, may be employed.

[0238] The fermentation medium may be selected from rich media, minimal media, and mineral salts media. In some embodiments, a minimal medium or a mineral salts medium is selected. In some embodiments, a mineral salts medium is selected.

[0239] Mineral salts media consists of mineral salts and a carbon source such as, e.g., glucose, sucrose, or glycerol. Examples of mineral salts media include, e.g., M9 medium, *Pseudomonas* medium (ATCC 179), and Davis and Mingioli medium (see, Davis, B. D., and Mingioli, E. S.,

1950, J. Bact. 60:17-28). The mineral salts used to make mineral salts media include those selected from among, e.g., potassium phosphates, ammonium sulfate or chloride, magnesium sulfate or chloride, and trace minerals such as calcium chloride, borate, and sulfates of iron, copper, manganese, and zinc. Typically, no organic nitrogen source, such as peptone, tryptone, amino acids, or a yeast extract, is included in a mineral salts medium. Instead, an inorganic nitrogen source is used and this may be selected from among, e.g., ammonium salts, aqueous ammonia, and gaseous ammonia. A mineral salts medium will typically contain glucose or glycerol as the carbon source. In comparison to mineral salts media, minimal media can also contain mineral salts and a carbon source, but can be supplemented with, e.g., low levels of amino acids, vitamins, peptones, or other ingredients, though these are added at very minimal levels. Suitable media for use in the methods of the present invention can be prepared using methods described in the literature, e.g., in U.S. Pat. No. 9,458,487 and U.S. Pat. No. 9,453,251. Details of cultivation procedures and mineral salts media useful in the methods of the present invention are described by Riesenberg, D et al., 1991, "High cell density cultivation of *Escherichia coli* at controlled specific growth rate," J. Biotechnol. 20 (1):17-27, incorporated by reference herein.

[0240] In embodiments, production can be achieved in bioreactor cultures. Cultures can be grown in, e.g., up to 2 L bioreactors containing a mineral salts medium, and maintained at 32 °C and pH 6.5 through the addition of ammonia. Dissolved oxygen can be maintained in excess through increases in agitation and flow of sparged air and oxygen into the fermentor. Glycerol can be delivered to the culture throughout the fermentation to maintain excess levels. In embodiments, these conditions are maintained until a target culture cell density, e.g., an optical density of 575 nm (A_{575}), for induction is reached and IPTG is added to initiate the target protein production. It is understood that the cell density at induction, the concentration of IPTG, pH, temperature, CaCl_2 concentration, dissolved oxygen flow rate, each can be varied to determine optimal conditions for expression. In embodiments, cell density at induction can be varied from A_{575} of 40 to 200 absorbance units (AU). IPTG concentrations can be varied in the range from 0.02 to 1.0 mM, pH from 5 to 7.5, temperature from 20 to 35° C, CaCl_2 concentration from 0 to 0.5 g/L, and the dissolved oxygen flow rate from 1 LPM (liters per minute) to 10 LPM. After 6-96 hours, the culture from each bioreactor can be harvested by centrifugation and the cell pellet frozen at -80° C. Samples can then be analyzed, e.g., by SDS-CGE, for product formation.

[0241] Fermentation may be performed at any scale. The expression systems according to the present invention are useful for recombinant protein expression at any scale. Thus, e.g.,

microliter-scale, milliliter scale, centiliter scale, and deciliter scale fermentation volumes may be used, and 1 Liter scale and larger fermentation volumes can be used.

[0242] In embodiments, the fermentation volume is at or above about 1 Liter. In embodiments, the fermentation volume is about 1 Liter to about 100 Liters. In embodiments, the fermentation volume is about 1 Liter, about 2 Liters, about 3 Liters about 4 Liters, about 5 Liters, about 6 Liters, about 7 Liters, about 8 Liters, about 9 Liters, or about 10 Liters. In embodiments, the fermentation volume is about 1 Liter to about 5 Liters, about 1 Liter to about 10 Liters, about 1 Liter to about 25 Liters, about 1 Liter to about 50 Liters, about 1 Liter to about 75 Liters, about 10 Liters to about 25 Liters, about 25 Liters to about 50 Liters, or about 50 Liters to about 100 Liters. In other embodiments, the fermentation volume is at or above 5 Liters, 10 Liters, 15 Liters, 20 Liters, 25 Liters, 50 Liters, 75 Liters, 100 Liters, 200 Liters, 250 Liters, 300 Liters, 500 Liters, 1,000 Liters, 2,000 Liters, 5,000 Liters, 10,000 Liters, or 50,000 Liters.

[0243] In general, the amount of a recombinant protein yielded by a larger culture volume, e.g., a 50 mL shake-flask culture, a 1 liter culture, or greater, is increased relative to that observed in a smaller culture volume, e.g., a 0.5 mL high-throughput screening culture. This can be due to not only the increase in culture size but, e.g., the ability to grow cells to a higher density in large-scale fermentation (e.g., as reflected by culture absorbance). For example, the volumetric yield from the same strain can increase up to ten-fold from HTP scale to large-scale fermentation. In embodiments, the volumetric yield observed for the same expression strain is 2-fold to 10-fold greater following large-scale fermentation than HTP scale growth. In embodiments, the yield observed for the same expression strain is 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 2-fold to 10-fold, 2-fold to 9-fold, 2-fold to 8-fold, 2-fold to 7-fold, 2-fold to 6-fold, 2-fold to 5-fold, 2-fold to 4-fold, 2-fold to 3-fold, 3-fold to 10-fold, 3-fold to 9-fold, 3-fold to 8-fold, 3-fold to 7-fold, 3-fold to 6-fold, 3-fold to 5-fold, 3-fold to 4-fold, 4-fold to 10-fold, 4-fold to 9-fold, 4-fold to 8-fold, 4-fold to 7-fold, 4-fold to 6-fold, 4-fold to 5-fold, 5-fold to 10-fold, 5-fold to 9-fold, 5-fold to 8-fold, 5-fold to 7-fold, 5-fold to 6-fold, 6-fold to 10-fold, 6-fold to 9-fold, 6-fold to 8-fold, 6-fold to 7-fold, 7-fold to 10-fold, 7-fold to 9-fold, 7-fold to 8-fold, 8-fold to 10-fold, 8-fold to 9-fold, 9-fold to 10-fold, greater following large-scale fermentation than following HTP-scale growth. See, e.g., Retallack, et al., 2012, "Reliable protein production in a *Pseudomonas fluorescens* expression system," Prot. Exp. and Purif. 81:157-165, incorporated herein by reference in its entirety.

[0244] Bacterial Growth Conditions

[0245] Suitable fermentation conditions useful in the methods of the provided invention can comprise growth at a temperature of about 4 deg C to about 42 deg C and a pH of about 5.7 to about 8.8. When an expression construct with a *lacZ* promoter is used, expression can be

induced by adding IPTG to a culture at a final concentration of about 0.01 mM to about 1.0 mM. In some embodiments, the fermentation conditions comprise induction of the inducible promoter at: an OD₅₇₅ of about 40 to about 200, a culture pH of about 5.5 to about 7.2, and a temperature of about 20 to about 34 deg C, fed batch. In some embodiments, the fermentation conditions comprise induction of the inducible promoter at: an OD₅₇₅ of about 80 to about 160, a culture pH of about 5.8 to about 7.0, a temperature of about 28 to about 33 deg C, fed batch. In some embodiments, the resulting recombinant protein titer is about 0.2 to about 5 g/L of cell culture.

[0246] The pH of the culture can be maintained using pH buffers and methods known to those of skill in the art. Control of pH during culturing also can be achieved using aqueous ammonia. In embodiments, the pH of the culture during growth, induction, and/or production phase is about 5 to about 8.8. In embodiments, the culture pH is about 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, or any range therein. In embodiments, the culture pH is about 5 to about 8.8. In embodiments, the culture pH is about 5 to about 5.5, about 5 to about 6, about 5 to about 6.5, about 5 to about 7, about 5 to about 7.5, about 5 to about 8, about 5 to about 8.5, about 5 to about 8.8, about 5.5 to about 6, about 5.5 to about 6.5, about 5.5 to about 7, about 5.5 to about 7.5, about 5.5 to about 8, about 5.5 to about 8.5, about 5.5 to about 8.8, about 6 to about 6.5, about 6 to about 7, about 6 to about 7.5, about 6 to about 8, about 6 to about 8.5, about 6 to about 8.8, about 6.5 to about 7, about 6.5 to about 7.5, about 6.5 to about 8, about 6.5 to about 8.5, about 6.5 to about 8.8, about 7 to about 7.5, about 7 to about 8, about 7 to about 8.5, about 7 to about 8.8, about 7.5 to about 8, about 7.5 to about 8.5, about 7.5 to about 8.8, about 8 to about 8.5, about 8 to about 8.8, or about 8.5 to about 8.8. In embodiments, the culture pH is about 5, about 5.5, about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, or about 8.8. In embodiments, the culture pH is at least about 5, about 5.5, about 6, about 6.5, about 7, about 7.5, about 8, or about 8.5. In embodiments, the culture pH is at most about 5.5, about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, or about 8.8. In embodiments, the culture pH is about 5.8 to about 7. In embodiments, the culture pH is about 5.8 to about 5.9, about 5.8 to about 6, about 5.8 to about 6.1, about 5.8 to about 6.2, about 5.8 to about 6.2, about 5.8 to about 6.4, about 5.8 to about 6.5, about 5.8 to about 6.6, about 5.8 to about 6.7, about 5.8 to about 6.8, about 5.8 to about 7, about 5.9 to about 6, about 5.9 to about 6.1, about 5.9 to about 6.2, about 5.9 to about 6.2, about 5.9 to about 6.4, about 5.9 to about 6.5, about 5.9 to about 6.6, about 5.9 to about 6.7, about 5.9 to about 6.8, about 5.9 to about 7, about 6 to about 6.1, about 6 to about 6.2, about 6 to about 6.2, about 6 to about 6.4, about 6 to about 6.5, about 6 to about 6.6, about 6 to about 6.7, about 6 to about 6.8, about 6 to about 7, about 6.1 to about 6.2, about 6.1 to about 6.2, about 6.1 to about 6.4, about 6.1 to about 6.5, about 6.1 to about 6.6, about 6.1 to about 6.7, about 6.1 to

about 6.8, about 6.1 to about 7, about 6.2 to about 6.2, about 6.2 to about 6.4, about 6.2 to about 6.5, about 6.2 to about 6.6, about 6.2 to about 6.7, about 6.2 to about 6.8, about 6.2 to about 7, about 6.2 to about 6.4, about 6.2 to about 6.5, about 6.2 to about 6.6, about 6.2 to about 6.7, about 6.2 to about 6.8, about 6.2 to about 7, about 6.4 to about 6.5, about 6.4 to about 6.6, about 6.4 to about 6.7, about 6.4 to about 6.8, about 6.4 to about 7, about 6.5 to about 6.6, about 6.5 to about 6.7, about 6.5 to about 6.8, about 6.5 to about 7, about 6.6 to about 6.7, about 6.6 to about 6.8, about 6.6 to about 7, about 6.7 to about 6.8, about 6.7 to about 7, or about 6.8 to about 7. In embodiments, the culture pH is about 5.8, about 5.9, about 6, about 6.1, about 6.2, about 6.2, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, or about 7. In embodiments, the culture pH is at least about 5.8, about 5.9, about 6, about 6.1, about 6.2, about 6.2, about 6.4, about 6.5, about 6.6, about 6.7, or about 6.8. In embodiments, the culture pH is at most about 5.9, about 6, about 6.1, about 6.2, about 6.2, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, or about 7. In some embodiments, the pH is about 6 to about 6.5. In some embodiments, the culture pH is about 6 to about 6.1, about 6 to about 6.2, about 6 to about 6.3, about 6 to about 6.4, about 6 to about 6.5, about 6.1 to about 6.2, about 6.1 to about 6.3, about 6.1 to about 6.4, about 6.1 to about 6.5, about 6.2 to about 6.3, about 6.2 to about 6.4, about 6.2 to about 6.5, about 6.3 to about 6.4, about 6.3 to about 6.5, or about 6.4 to about 6.5. In some embodiments, the culture pH is about 6, about 6.1, about 6.2, about 6.3, about 6.4, or about 6.5. In some embodiments, the culture pH is at least about 6, about 6.1, about 6.2, about 6.3, or about 6.4. In some embodiments, the culture pH is at most about 6.1, about 6.2, about 6.3, about 6.4, or about 6.5.

[0247] In embodiments, the growth temperature of the culture during growth, induction, and/or production phase is maintained at about 4 °C to about 42 °C. In embodiments, the growth temperature is about 4 °C, about 5 °C, about 6 °C, about 7 °C, about 8 °C, about 9 °C, about 10 °C, about 11 °C, about 12 °C, about 13 °C, about 14 °C, about 15 °C, about 16 °C, about 17 °C, about 18 °C, about 19 °C, about 20 °C, about 21 °C, about 22 °C, about 23 °C, about 24 °C, about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, about 37 °C, about 38 °C, about 39 °C, about 40 °C, about 41 °C, about 42 °C, or any range therein. In embodiments, the growth temperature is about 25 °C to about 35 °C. In embodiments, the growth temperature is about 25 °C to about 35 °C. In embodiments, the growth temperature is about 25 °C to about 26 °C, about 25 °C to about 27 °C, about 25 °C to about 28 °C, about 25 °C to about 29 °C, about 25 °C to about 30 °C, about 25 °C to about 31 °C, about 25 °C to about 32 °C, about 25 °C to about 33 °C, about 25 °C to about 34 °C, about 25 °C to about 35 °C, about 26 °C to about 27 °C, about 26 °C to about 28 °C, about 26 °C to about 29 °C, about 26 °C to about 30 °C, about 26 °C to about 31 °C, about 26 °C to about 32 °C, about 26 °C to about 33 °C, about 26 °C to about 34 °C, about 26

°C to about 35 °C, about 27 °C to about 28 °C, about 27 °C to about 29 °C, about 27 °C to about 30 °C, about 27 °C to about 31 °C, about 27 °C to about 32 °C, about 27 °C to about 33 °C, about 27 °C to about 34 °C, about 27 °C to about 35 °C, about 28 °C to about 29 °C, about 28 °C to about 30 °C, about 28 °C to about 31 °C, about 28 °C to about 32 °C, about 28 °C to about 33 °C, about 28 °C to about 34 °C, about 28 °C to about 35 °C, about 29 °C to about 30 °C, about 29 °C to about 31 °C, about 29 °C to about 32 °C, about 29 °C to about 33 °C, about 29 °C to about 34 °C, about 29 °C to about 35 °C, about 30 °C to about 31 °C, about 30 °C to about 32 °C, about 30 °C to about 33 °C, about 30 °C to about 34 °C, about 30 °C to about 35 °C, about 31 °C to about 32 °C, about 31 °C to about 33 °C, about 31 °C to about 34 °C, about 31 °C to about 35 °C, about 32 °C to about 33 °C, about 32 °C to about 34 °C, about 32 °C to about 35 °C, about 33 °C to about 34 °C, about 33 °C to about 35 °C, or about 34 °C to about 35 °C. In embodiments, the growth temperature is about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, or about 35 °C. In embodiments, the growth temperature is at least about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, or about 34 °C. In embodiments, the growth temperature is at most about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, or about 35 °C.

[0248] In embodiments, the temperature is changed during culturing. In embodiments, the temperature is maintained at about 30 °C to about 32 °C before an agent, e.g., IPTG, is added to the culture to induce expression from the construct, and after adding the induction agent, the temperature is reduced to about 25 °C to about 28 °C. In embodiments, the temperature is maintained at about 30 °C before an agent, e.g., IPTG, is added to the culture to induce expression from the construct, and after adding the induction agent, the temperature is reduced to about 25 °C.

[0249] As described elsewhere herein, inducible promoters can be used in the expression construct to control expression of the recombinant protein of interest, e.g., a *lac* promoter. In the case of the *lac* promoter derivatives or family members, e.g., the *tac* promoter, the effector compound is an inducer, such as a gratuitous inducer like IPTG. In embodiments, a *lac* promoter derivative is used, and recombinant protein expression is induced by the addition of IPTG to a final concentration of about 0.01 mM to about 1.0 mM, when the cell density has reached a level identified by an OD₅₇₅ of about 80 to about 300. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is about 80 to about 300. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is about 80 to about 100, about 80 to about 120, about 80 to about 140, about 80 to about 160, about 80 to about 180, about 80 to about 200, about 80 to about 220, about 80 to about 240, about 80 to about 260, about 80 to

about 280, about 80 to about 300, about 100 to about 120, about 100 to about 140, about 100 to about 160, about 100 to about 180, about 100 to about 200, about 100 to about 220, about 100 to about 240, about 100 to about 260, about 100 to about 280, about 100 to about 300, about 120 to about 140, about 120 to about 160, about 120 to about 180, about 120 to about 200, about 120 to about 220, about 120 to about 240, about 120 to about 260, about 120 to about 280, about 120 to about 300, about 140 to about 160, about 140 to about 180, about 140 to about 200, about 140 to about 220, about 140 to about 240, about 140 to about 260, about 140 to about 280, about 140 to about 300, about 160 to about 180, about 160 to about 200, about 160 to about 220, about 160 to about 240, about 160 to about 260, about 160 to about 280, about 160 to about 300, about 180 to about 200, about 180 to about 220, about 180 to about 240, about 180 to about 260, about 180 to about 280, about 180 to about 300, about 200 to about 220, about 200 to about 240, about 200 to about 260, about 200 to about 280, about 200 to about 300, about 220 to about 240, about 220 to about 260, about 220 to about 280, about 220 to about 300, about 240 to about 260, about 240 to about 280, about 240 to about 300, about 260 to about 280, about 260 to about 300, or about 280 to about 300. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is about 80, about 100, about 120, about 140, about 160, about 180, about 200, about 220, about 240, about 260, about 280, or about 300. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is at least about 80, about 100, about 120, about 140, about 160, about 180, about 200, about 220, about 240, about 260, or about 280. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is at most about 100, about 120, about 140, about 160, about 180, about 200, about 220, about 240, about 260, about 280, or about 300. In some embodiments, the induction OD₅₇₅ is about 80-160. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is about 80 to about 160. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is about 80 to about 90, about 80 to about 100, about 80 to about 110, about 80 to about 120, about 80 to about 130, about 80 to about 140, about 80 to about 150, about 80 to about 160, about 90 to about 100, about 90 to about 110, about 90 to about 120, about 90 to about 130, about 90 to about 140, about 90 to about 150, about 90 to about 160, about 100 to about 110, about 100 to about 120, about 100 to about 130, about 100 to about 140, about 100 to about 150, about 100 to about 160, about 110 to about 120, about 110 to about 130, about 110 to about 140, about 110 to about 150, about 110 to about 160, about 120 to about 130, about 120 to about 140, about 120 to about 150, about 120 to about 160, about 130 to about 140, about 130 to about 150, about 130 to about 160, about 140 to about 150, about 140 to about 160, or about 150 to about 160. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is about 80, about 90, about 100, about 110, about 120,

about 130, about 140, about 150, or about 160. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is at least about 80, about 90, about 100, about 110, about 120, about 130, about 140, or about 150. In some embodiments, the OD₅₇₅ at the time of culture induction for the recombinant protein is at most about 90, about 100, about 110, about 120, about 130, about 140, about 150, or about 160.

[0250] The cell density can be measured by other methods and expressed in other units, e.g., in cells per unit volume. For example, an OD₅₇₅ of about 40 to about 160 of a *P. fluorescens* culture is equivalent to approximately 4×10^{10} to about 1.6×10^{11} colony forming units per mL or 17.5 to 70 g/L dry cell weight. In embodiments, the cell density at the time of culture induction is equivalent to the cell density as specified herein by the absorbance at OD₅₇₅, regardless of the method used for determining cell density or the units of measurement. One of skill in the art will know how to make the appropriate conversion for any cell culture.

[0251] In some embodiments, the final IPTG concentration of the culture is about 0.01 mM to about 1 mM. In some embodiments, the final IPTG concentration of the culture is about 0.01 mM to about 0.02 mM, about 0.01 mM to about 0.03 mM, about 0.01 mM to about 0.05 mM, about 0.01 mM to about 0.06 mM, about 0.01 mM to about 0.07 mM, about 0.01 mM to about 0.08 mM, about 0.01 mM to about 0.09 mM, about 0.01 mM to about 0.1 mM, about 0.01 mM to about 0.2 mM, about 0.01 mM to about 0.5 mM, about 0.01 mM to about 1 mM, about 0.02 mM to about 0.03 mM, about 0.02 mM to about 0.05 mM, about 0.02 mM to about 0.06 mM, about 0.02 mM to about 0.07 mM, about 0.02 mM to about 0.08 mM, about 0.02 mM to about 0.09 mM, about 0.02 mM to about 0.1 mM, about 0.02 mM to about 0.2 mM, about 0.02 mM to about 0.5 mM, about 0.02 mM to about 1 mM, about 0.03 mM to about 0.05 mM, about 0.03 mM to about 0.06 mM, about 0.03 mM to about 0.07 mM, about 0.03 mM to about 0.08 mM, about 0.03 mM to about 0.09 mM, about 0.03 mM to about 0.1 mM, about 0.03 mM to about 0.2 mM, about 0.03 mM to about 0.5 mM, about 0.03 mM to about 1 mM, about 0.05 mM to about 0.06 mM, about 0.05 mM to about 0.07 mM, about 0.05 mM to about 0.08 mM, about 0.05 mM to about 0.09 mM, about 0.05 mM to about 0.1 mM, about 0.05 mM to about 0.2 mM, about 0.05 mM to about 0.5 mM, about 0.05 mM to about 1 mM, about 0.06 mM to about 0.07 mM, about 0.06 mM to about 0.08 mM, about 0.06 mM to about 0.09 mM, about 0.06 mM to about 0.1 mM, about 0.06 mM to about 0.2 mM, about 0.06 mM to about 0.5 mM, about 0.06 mM to about 1 mM, about 0.07 mM to about 0.08 mM, about 0.07 mM to about 0.09 mM, about 0.07 mM to about 0.1 mM, about 0.07 mM to about 0.2 mM, about 0.07 mM to about 0.5 mM, about 0.07 mM to about 1 mM, about 0.08 mM to about 0.09 mM, about 0.08 mM to about 0.1 mM, about 0.08 mM to about 0.2 mM, about 0.08 mM to about 0.5 mM, about 0.08 mM to about 1 mM, about 0.09 mM to about 0.1 mM, about 0.09 mM to about 0.2 mM, about 0.09 mM to about 0.5

mM, about 0.09 mM to about 1 mM, about 0.1 mM to about 0.2 mM, about 0.1 mM to about 0.5 mM, about 0.1 mM to about 1 mM, about 0.2 mM to about 0.5 mM, about 0.2 mM to about 1 mM, or about 0.5 mM to about 1 mM. In some embodiments, the final IPTG concentration of the culture is about 0.01 mM, about 0.02 mM, about 0.03 mM, about 0.05 mM, about 0.06 mM, about 0.07 mM, about 0.08 mM, about 0.09 mM, about 0.1 mM, about 0.2 mM, about 0.5 mM, or about 1 mM. In some embodiments, the final IPTG concentration of the culture is at least about 0.01 mM, about 0.02 mM, about 0.03 mM, about 0.05 mM, about 0.06 mM, about 0.07 mM, about 0.08 mM, about 0.09 mM, about 0.1 mM, about 0.2 mM, or about 0.5 mM. In some embodiments, the final IPTG concentration of the culture is at most about 0.02 mM, about 0.03 mM, about 0.05 mM, about 0.06 mM, about 0.07 mM, about 0.08 mM, about 0.09 mM, about 0.1 mM, about 0.2 mM, about 0.5 mM, or about 1 mM. In some embodiments, the final IPTG concentration of the culture is about 0.08 mM to about 0.3 mM. In some embodiments, the final IPTG concentration of the culture is about 0.08 mM to about 0.09 mM, about 0.08 mM to about 0.1 mM, about 0.08 mM to about 0.125 mM, about 0.08 mM to about 0.15 mM, about 0.08 mM to about 0.175 mM, about 0.08 mM to about 0.2 mM, about 0.08 mM to about 0.225 mM, about 0.08 mM to about 0.25 mM, about 0.08 mM to about 0.275 mM, about 0.08 mM to about 0.3 mM, about 0.09 mM to about 0.1 mM, about 0.09 mM to about 0.125 mM, about 0.09 mM to about 0.15 mM, about 0.09 mM to about 0.175 mM, about 0.09 mM to about 0.2 mM, about 0.09 mM to about 0.225 mM, about 0.09 mM to about 0.25 mM, about 0.09 mM to about 0.275 mM, about 0.09 mM to about 0.3 mM, about 0.1 mM to about 0.125 mM, about 0.1 mM to about 0.15 mM, about 0.1 mM to about 0.175 mM, about 0.1 mM to about 0.2 mM, about 0.1 mM to about 0.225 mM, about 0.1 mM to about 0.25 mM, about 0.1 mM to about 0.275 mM, about 0.1 mM to about 0.3 mM, about 0.125 mM to about 0.15 mM, about 0.125 mM to about 0.175 mM, about 0.125 mM to about 0.2 mM, about 0.125 mM to about 0.225 mM, about 0.125 mM to about 0.25 mM, about 0.125 mM to about 0.275 mM, about 0.125 mM to about 0.3 mM, about 0.15 mM to about 0.175 mM, about 0.15 mM to about 0.2 mM, about 0.15 mM to about 0.225 mM, about 0.15 mM to about 0.25 mM, about 0.15 mM to about 0.275 mM, about 0.15 mM to about 0.3 mM, about 0.175 mM to about 0.2 mM, about 0.175 mM to about 0.225 mM, about 0.175 mM to about 0.25 mM, about 0.175 mM to about 0.275 mM, about 0.175 mM to about 0.3 mM, about 0.2 mM to about 0.225 mM, about 0.2 mM to about 0.25 mM, about 0.2 mM to about 0.275 mM, about 0.2 mM to about 0.3 mM, about 0.225 mM to about 0.25 mM, about 0.225 mM to about 0.275 mM, about 0.225 mM to about 0.3 mM, about 0.25 mM to about 0.275 mM, about 0.25 mM to about 0.3 mM, or about 0.275 mM to about 0.3 mM. In some embodiments, the final IPTG concentration of the culture is about 0.08 mM, about 0.09 mM, about 0.1 mM, about 0.125 mM, about 0.15 mM, about 0.175 mM, about 0.2 mM, about 0.225 mM, about 0.25 mM, about

0.275 mM, or about 0.3 mM. In some embodiments, the final IPTG concentration of the culture is at least about 0.08 mM, about 0.09 mM, about 0.1 mM, about 0.125 mM, about 0.15 mM, about 0.175 mM, about 0.2 mM, about 0.225 mM, about 0.25 mM, or about 0.275 mM. In some embodiments, the final IPTG concentration of the culture is at most about 0.09 mM, about 0.1 mM, about 0.125 mM, about 0.15 mM, about 0.175 mM, about 0.2 mM, about 0.225 mM, about 0.25 mM, about 0.275 mM, or about 0.3 mM.

[0252] In embodiments wherein a non-lac type promoter is used, as described herein and in the literature, other inducers or effectors can be used. In one embodiment, the promoter is a constitutive promoter.

[0253] After adding and inducing agent, cultures can be grown for a period of time, for example about 24 hours, during which time the recombinant protein is expressed (production phase). After adding an inducing agent, a culture can be grown for about 1 hr, about 2 hr, about 3 hr, about 4 hr, about 5 hr, about 6 hr, about 7 hr, about 8 hr, about 9 hr, about 10 hr, about 11 hr, about 12 hr, about 13 hr, about 14 hr, about 15 hr, about 16 hr, about 17 hr, about 18 hr, about 19 hr, about 20 hr, about 21 hr, about 22 hr, about 23 hr, about 24 hr, about 36 hr, or about 48 hr. After an inducing agent is added to a culture, the culture can be grown for about 1 to 48 hr, about 1 to 24 hr, about 1 to 8 hr, about 10 to 24 hr, about 15 to 24 hr, or about 20 to 24 hr. Cell cultures can be concentrated by centrifugation, and the culture pellet resuspended in a buffer or solution appropriate for the subsequent lysis procedure.

[0254] In some embodiments a constant feed is used. In some embodiments, a fed-batch format is used. In some embodiments, the feed is glycerol or glucose. In some embodiments the feed bolus is about 10 g/L to about 50 g/L. In some embodiments the feed bolus is about 10 g/L to about 15 g/L, about 10 g/L to about 20 g/L, about 10 g/L to about 25 g/L, about 10 g/L to about 30 g/L, about 10 g/L to about 35 g/L, about 10 g/L to about 40 g/L, about 10 g/L to about 45 g/L, about 10 g/L to about 50 g/L, about 15 g/L to about 20 g/L, about 15 g/L to about 25 g/L, about 15 g/L to about 30 g/L, about 15 g/L to about 35 g/L, about 15 g/L to about 40 g/L, about 15 g/L to about 45 g/L, about 15 g/L to about 50 g/L, about 20 g/L to about 25 g/L, about 20 g/L to about 30 g/L, about 20 g/L to about 35 g/L, about 20 g/L to about 40 g/L, about 20 g/L to about 45 g/L, about 20 g/L to about 50 g/L, about 25 g/L to about 30 g/L, about 25 g/L to about 35 g/L, about 25 g/L to about 40 g/L, about 25 g/L to about 45 g/L, about 25 g/L to about 50 g/L, about 30 g/L to about 35 g/L, about 30 g/L to about 40 g/L, about 30 g/L to about 45 g/L, about 30 g/L to about 50 g/L, about 35 g/L to about 40 g/L, about 35 g/L to about 45 g/L, about 35 g/L to about 50 g/L, about 40 g/L to about 45 g/L, about 40 g/L to about 50 g/L, or about 45 g/L to about 50 g/L. In some embodiments the feed bolus is about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 35 g/L, about 40 g/L, about 45 g/L, or about 50 g/L. In some

embodiments the feed bolus is at least about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 35 g/L, about 40 g/L, or about 45 g/L. In some embodiments the feed bolus is at most about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 35 g/L, about 40 g/L, about 45 g/L, or about 50 g/L.

[0255] In embodiments, cells are disrupted using equipment for high pressure mechanical cell disruption (which are available commercially, e.g., Microfluidics Micro fluidizer, Constant Cell Disruptor, Niro-Soavi homogenizer or APV-Gaulin homogenizer). Cells expressing the recombinant protein can be disrupted, for example, using sonication. Any appropriate method known in the art for lysing cells can be used to release the soluble fraction. For example, in embodiments, chemical and/or enzymatic cell lysis reagents, such as cell-wall lytic enzyme and EDTA, can be used. Use of frozen or previously stored cultures is also contemplated in the methods of the invention. Cultures can be OD-normalized prior to lysis. For example, cells can be normalized to an OD₆₀₀ of about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20.

[0256] Centrifugation can be performed using any appropriate equipment and method. Centrifugation of cell culture or lysate for the purposes of separating a soluble fraction from an insoluble fraction is well-known in the art. For example, lysed cells can be centrifuged at 20,800×g for 20 minutes (at 4°C), and the supernatants removed using manual or automated liquid handling. The cell pellet obtained by centrifugation of cell culture, or the insoluble fraction obtained by centrifugation of cell lysate, can be resuspended in a buffered solution. Resuspension of the cell pellet or insoluble fraction can be carried out using, e.g., equipment such as impellers connected to an overhead mixer, magnetic stir-bars, rocking shakers, etc.

[0257] A “soluble fraction,” i.e., the soluble supernatant obtained after centrifugation of a lysate, and an “insoluble fraction,” i.e., the pellet obtained after centrifugation of a lysate, result from lysing and centrifuging the cultures.

High Throughput Screens

[0258] In embodiments, a high throughput screen is conducted to determine optimal conditions for expressing a recombinant protein of interest. Conditions that can be varied in the screen include, for example, the host cell, genetic background of the host cell (e.g., as described in detail herein), type of promoter in an expression construct, type of secretion leader fused to the encoded polypeptide or protein of interest, temperature of growth, OD of induction when an inducible promoter is used, amount of inducer added (e.g. amount of IPTG used for induction when a lacZ promoter or derivative thereof is used), duration of protein induction, temperature of growth following addition of an inducing agent to a culture, rate of agitation of culture, method of selection for plasmid maintenance, volume of culture in a vessel, and method of cell lysing.

[0259] In some embodiments, a library (or “array”) of host strains is provided, wherein each strain (or “population of host cells”) in the library has been genetically modified to modulate the expression of one or more target genes in the host cell. An “optimal host strain” or “optimal expression system” may be identified or selected based on the quantity, quality, and/or location of the expressed protein of interest compared to other populations of phenotypically distinct host cells in the array. Thus, an optimal host strain is the strain that produces the recombinant protein of interest according to a desired specification. While the desired specification will vary depending on the polypeptide being produced, the specification includes the quality and/or quantity of protein, whether the protein is sequestered or secreted, protein folding, and the like. For example, the optimal host strain or optimal expression system produces a yield, characterized by the amount of soluble recombinant protein, the amount of recoverable recombinant protein, the amount of properly processed recombinant protein, the amount of properly folded recombinant protein, the amount of active recombinant protein, and/or the total amount of the recombinant protein of interest, of a certain absolute level or a certain level relative to that produced by a control or indicator strain, i.e., a strain used for comparison. Methods of screening microbial hosts to identify strains with improved yield and/or quality in the expression of recombinant proteins are described, for example, in U.S. Pat. Nos. 9,394,571 and 9,580,719.

[0260] Protein Analysis

[0261] A recombinant protein of interest produced according to the methods of the present invention may be of high quality, e.g., active, soluble, and/or intact; produced at a high yield or titer; or any combination thereof. In some embodiments, a recombinant protein of interest is produced by a recombinant gram-negative bacterial host cell according to the methods of the present invention at higher quality and/or higher yield when compared to those observed with a control host cell. In some embodiments, a recombinant gram-negative bacterial host cell of the present invention grows to a higher cell density than a control host cell.

[0262] In embodiments, recombinant proteins of interest produced by the methods provided herein are analyzed with regard to yield, solubility, activity, and degradation (e.g., by measuring intact protein). A recombinant protein of interest can be analyzed by any appropriate method known to those of skill in the art. The “solubility” and “activity” of a protein, though related qualities, are generally determined by different means. Solubility of a protein, particularly a hydrophobic protein, indicates that hydrophobic amino acid residues are properly located on the inside of the folded protein. Protein activity, which is often evaluated using different methods, e.g., as described below, is another indicator of proper protein conformation.

[0263] In some embodiments, a recombinant protein of interest is analyzed by biolayer interferometry, SDS-PAGE, Western blot, Far Western blot, ELISA, absorbance, or mass

spectrometry (e.g., tandem mass spectrometry). In some embodiments, the concentration and/or amounts of polypeptides or proteins of interest generated are determined, for example, by Bradford assay, absorbance, Coomassie staining, mass spectrometry, etc. Protein yield and fragmentation in the insoluble and soluble fractions can be analyzed by methods known to those of skill in the art, for example, by capillary gel electrophoresis (CGE), SDS-PAGE, and Western blot analysis. Soluble fractions also can be evaluated, for example, using biolayer interferometry. Protein activity may be measured by any known method as appropriate for the recombinant protein of interest. For a recombinant protein of interest that is a binding protein, this may comprise measuring its binding to a target ligand, e.g., TNF-alpha, or any other target, by any known method.

[0264] Useful measures of protein yield include, e.g., the amount of recombinant protein per culture volume (e.g., concentration, which may be expressed in grams or milligrams of protein/liter of culture), percent or fraction of recombinant protein measured in the insoluble pellet obtained after lysis (e.g., amount of recombinant protein in extract supernatant/amount of protein in insoluble fraction), percent or fraction of active protein (e.g., amount of active protein/amount protein used in the assay), percent or fraction of total cell protein (tcp), amount of protein/cell, and percent dry biomass. A measure as used herein may refer to that determined for a large-scale fermentation culture.

[0265] In some embodiments, a recombinant gram-negative bacterial host cell of the invention grows to an increased cell density in culture than a control cell, under substantially the same growth conditions. In some embodiments, the increase in cell density relative to the control cell is about 2-fold to about 15-fold. In some embodiments, the increase in cell density relative to the control cell is about 2 fold to about 3 fold, about 2 fold to about 4 fold, about 2 fold to about 5 fold, about 2 fold to about 6 fold, about 2 fold to about 7 fold, about 2 fold to about 8 fold, about 2 fold to about 9 fold, about 2 fold to about 10 fold, about 2 fold to about 11 fold, about 2 fold to about 12 fold, about 2 fold to about 15 fold, about 3 fold to about 4 fold, about 3 fold to about 5 fold, about 3 fold to about 6 fold, about 3 fold to about 7 fold, about 3 fold to about 8 fold, about 3 fold to about 9 fold, about 3 fold to about 10 fold, about 3 fold to about 11 fold, about 3 fold to about 12 fold, about 3 fold to about 15 fold, about 4 fold to about 5 fold, about 4 fold to about 6 fold, about 4 fold to about 7 fold, about 4 fold to about 8 fold, about 4 fold to about 9 fold, about 4 fold to about 10 fold, about 4 fold to about 11 fold, about 4 fold to about 12 fold, about 4 fold to about 15 fold, about 5 fold to about 6 fold, about 5 fold to about 7 fold, about 5 fold to about 8 fold, about 5 fold to about 9 fold, about 5 fold to about 10 fold, about 5 fold to about 11 fold, about 5 fold to about 12 fold, about 5 fold to about 15 fold, about 6 fold to about 7 fold, about 6 fold to about 8 fold, about 6 fold to about 9 fold, about 6 fold to about 10 fold, about 6 fold to

about 11 fold, about 6 fold to about 12 fold, about 6 fold to about 15 fold, about 7 fold to about 8 fold, about 7 fold to about 9 fold, about 7 fold to about 10 fold, about 7 fold to about 11 fold, about 7 fold to about 12 fold, about 7 fold to about 15 fold, about 8 fold to about 9 fold, about 8 fold to about 10 fold, about 8 fold to about 11 fold, about 8 fold to about 12 fold, about 8 fold to about 15 fold, about 9 fold to about 10 fold, about 9 fold to about 11 fold, about 9 fold to about 12 fold, about 9 fold to about 15 fold, about 10 fold to about 11 fold, about 10 fold to about 12 fold, about 10 fold to about 15 fold, about 11 fold to about 12 fold, about 11 fold to about 15 fold, or about 12 fold to about 15 fold. In some embodiments, the increase in cell density relative to the control cell is about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 11 fold, about 12 fold, or about 15 fold. In some embodiments, the increase in cell density relative to the control cell is at least about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 11 fold, or about 12 fold. In some embodiments, the increase in cell density relative to the control cell is at most about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 11 fold, about 12 fold, or about 15 fold.

[0266] In some embodiments, a recombinant gram-negative bacterial host cell of the invention produces an increased yield of high-quality recombinant protein relative to a control cell. In some embodiments, the increased yield relative to the control cell is about 2-fold to about 100-fold. In some embodiments, the increased yield relative to the control cell is about 2 fold to about 5 fold, about 2 fold to about 10 fold, about 2 fold to about 20 fold, about 2 fold to about 30 fold, about 2 fold to about 40 fold, about 2 fold to about 50 fold, about 2 fold to about 60 fold, about 2 fold to about 70 fold, about 2 fold to about 80 fold, about 2 fold to about 90 fold, about 2 fold to about 100 fold, about 5 fold to about 10 fold, about 5 fold to about 20 fold, about 5 fold to about 30 fold, about 5 fold to about 40 fold, about 5 fold to about 50 fold, about 5 fold to about 60 fold, about 5 fold to about 70 fold, about 5 fold to about 80 fold, about 5 fold to about 90 fold, about 5 fold to about 100 fold, about 10 fold to about 20 fold, about 10 fold to about 30 fold, about 10 fold to about 40 fold, about 10 fold to about 50 fold, about 10 fold to about 60 fold, about 10 fold to about 70 fold, about 10 fold to about 80 fold, about 10 fold to about 90 fold, about 10 fold to about 100 fold, about 20 fold to about 30 fold, about 20 fold to about 40 fold, about 20 fold to about 50 fold, about 20 fold to about 60 fold, about 20 fold to about 70 fold, about 20 fold to about 80 fold, about 20 fold to about 90 fold, about 20 fold to about 100 fold, about 30 fold to about 40 fold, about 30 fold to about 50 fold, about 30 fold to about 60 fold, about 30 fold to about 70 fold, about 30 fold to about 80 fold, about 30 fold to about 90 fold, about 30 fold to about 100 fold, about 40 fold to about 50 fold, about 40 fold to about 60 fold, about 40 fold to about 70 fold, about 40 fold to about 80 fold, about 40 fold to about 90 fold, about 40 fold to

about 100 fold, about 50 fold to about 60 fold, about 50 fold to about 70 fold, about 50 fold to about 80 fold, about 50 fold to about 90 fold, about 50 fold to about 100 fold, about 60 fold to about 70 fold, about 60 fold to about 80 fold, about 60 fold to about 90 fold, about 60 fold to about 100 fold, about 70 fold to about 80 fold, about 70 fold to about 90 fold, about 70 fold to about 100 fold, about 80 fold to about 90 fold, about 80 fold to about 100 fold, or about 90 fold to about 100 fold. In some embodiments, the increased yield relative to the control cell is about 2 fold, about 5 fold, about 10 fold, about 20 fold, about 30 fold, about 40 fold, about 50 fold, about 60 fold, about 70 fold, about 80 fold, about 90 fold, or about 100 fold. In some embodiments, the increased yield relative to the control cell is at least about 2 fold, about 5 fold, about 10 fold, about 20 fold, about 30 fold, about 40 fold, about 50 fold, about 60 fold, about 70 fold, about 80 fold, or about 90 fold. In some embodiments, the increased yield relative to the control cell is at most about 5 fold, about 10 fold, about 20 fold, about 30 fold, about 40 fold, about 50 fold, about 60 fold, about 70 fold, about 80 fold, about 90 fold, or about 100 fold.

[0267] Any suitable control cell may be selected by one of skill in the art for comparison with the recombinant gram-negative bacterial host cell. In some embodiments, the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from: (i) a recombinant gram-negative bacterial host cell deficient in a first protease activity and a second protease activity as described herein, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional; (ii) a recombinant gram-negative bacterial host cell deficient in the first protease activity, the second protease activity, and an additional protease activity as described herein, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and (iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell.

[0268] In embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 20% to about 90% total cell protein. In certain embodiments, the yield of active, soluble, and/or intact polypeptide or protein of interest is about 20% total cell protein, about 25% total cell protein, about 30% total cell protein, about 31% total

cell protein, about 32% total cell protein, about 33% total cell protein, about 34% total cell protein, about 35% total cell protein, about 36% total cell protein, about 37% total cell protein, about 38% total cell protein, about 39% total cell protein, about 40% total cell protein, about 41% total cell protein, about 42% total cell protein, about 43% total cell protein, about 44% total cell protein, about 45% total cell protein, about 46% total cell protein, about 47% total cell protein, about 48% total cell protein, about 49% total cell protein, about 50% total cell protein, about 51% total cell protein, about 52% total cell protein, about 53% total cell protein, about 54% total cell protein, about 55% total cell protein, about 56% total cell protein, about 57% total cell protein, about 58% total cell protein, about 59% total cell protein, about 60% total cell protein, about 65% total cell protein, about 70% total cell protein, about 75% total cell protein, about 80% total cell protein, about 85% total cell protein, or about 90% total cell protein. In some embodiments, the yield of active, soluble, and/or intact recombinant protein of interest is about 20% to about 25% total cell protein, about 20% to about 30% total cell protein, about 20% to about 35% total cell protein, about 20% to about 40% total cell protein, about 20% to about 45% total cell protein, about 20% to about 50% total cell protein, about 20% to about 55% total cell protein, about 20% to about 60% total cell protein, about 20% to about 65% total cell protein, about 20% to about 70% total cell protein, about 20% to about 75% total cell protein, about 20% to about 80% total cell protein, about 20% to about 85% total cell protein, about 20% to about 90% total cell protein, about 25% to about 90% total cell protein, about 30% to about 90% total cell protein, about 35% to about 90% total cell protein, about 40% to about 90% total cell protein, about 45% to about 90% total cell protein, about 50% to about 90% total cell protein, about 55% to about 90% total cell protein, about 60% to about 90% total cell protein, about 65% to about 90% total cell protein, about 70% to about 90% total cell protein, about 75% to about 90% total cell protein, about 80% to about 90% total cell protein, about 85% to about 90% total cell protein, about 31% to about 60% total cell protein, about 35% to about 60% total cell protein, about 40% to about 60% total cell protein, about 45% to about 60% total cell protein, about 50% to about 60% total cell protein, about 55% to about 60% total cell protein, about 31% to about 55% total cell protein, about 31% to about 50% total cell protein, about 31% to about 45% total cell protein, about 31% to about 40% total cell protein, about 31% to about 35% total cell protein, about 35% to about 55% total cell protein, or about 40% to about 50% total cell protein.

[0269] In embodiments, the methods herein are used to obtain a yield (which may be referred to as a titer when expressed as a concentration) of active, soluble, and/or intact recombinant protein of interest of about 1 gram per liter to about 50 grams per liter. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of

interest of about 0.1 g/L to about 50 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.1 g/L to about 1 g/L, about 0.1 g/L to about 5 g/L, about 0.1 g/L to about 10 g/L, about 0.1 g/L to about 15 g/L, about 0.1 g/L to about 20 g/L, about 0.1 g/L to about 25 g/L, about 0.1 g/L to about 30 g/L, about 0.1 g/L to about 35 g/L, about 0.1 g/L to about 40 g/L, about 0.1 g/L to about 45 g/L, about 0.1 g/L to about 50 g/L, about 1 g/L to about 5 g/L, about 1 g/L to about 10 g/L, about 1 g/L to about 15 g/L, about 1 g/L to about 20 g/L, about 1 g/L to about 25 g/L, about 1 g/L to about 30 g/L, about 1 g/L to about 35 g/L, about 1 g/L to about 40 g/L, about 1 g/L to about 45 g/L, about 1 g/L to about 50 g/L, about 5 g/L to about 10 g/L, about 5 g/L to about 15 g/L, about 5 g/L to about 20 g/L, about 5 g/L to about 25 g/L, about 5 g/L to about 30 g/L, about 5 g/L to about 35 g/L, about 5 g/L to about 40 g/L, about 5 g/L to about 45 g/L, about 5 g/L to about 50 g/L, about 10 g/L to about 15 g/L, about 10 g/L to about 20 g/L, about 10 g/L to about 25 g/L, about 10 g/L to about 30 g/L, about 10 g/L to about 35 g/L, about 10 g/L to about 40 g/L, about 10 g/L to about 45 g/L, about 10 g/L to about 50 g/L, about 15 g/L to about 20 g/L, about 15 g/L to about 25 g/L, about 15 g/L to about 30 g/L, about 15 g/L to about 35 g/L, about 15 g/L to about 40 g/L, about 15 g/L to about 45 g/L, about 15 g/L to about 50 g/L, about 20 g/L to about 25 g/L, about 20 g/L to about 30 g/L, about 20 g/L to about 35 g/L, about 20 g/L to about 40 g/L, about 20 g/L to about 45 g/L, about 20 g/L to about 50 g/L, about 25 g/L to about 30 g/L, about 25 g/L to about 35 g/L, about 25 g/L to about 40 g/L, about 25 g/L to about 45 g/L, about 25 g/L to about 50 g/L, about 30 g/L to about 35 g/L, about 30 g/L to about 40 g/L, about 30 g/L to about 45 g/L, about 30 g/L to about 50 g/L, about 35 g/L to about 40 g/L, about 35 g/L to about 45 g/L, about 35 g/L to about 50 g/L, about 40 g/L to about 45 g/L, about 40 g/L to about 50 g/L, or about 45 g/L to about 50 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.1 g/L, about 1 g/L, about 5 g/L, about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 35 g/L, about 40 g/L, about 45 g/L, or about 50 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of at least about 0.1 g/L, about 1 g/L, about 5 g/L, about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 35 g/L, about 40 g/L, or about 45 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of at most about 1 g/L, about 5 g/L, about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 35 g/L, about 40 g/L, about 45 g/L, or about 50 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.1 g/L to about 10 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.1 g/L to

about 0.5 g/L, about 0.1 g/L to about 1 g/L, about 0.1 g/L to about 2 g/L, about 0.1 g/L to about 3 g/L, about 0.1 g/L to about 4 g/L, about 0.1 g/L to about 5 g/L, about 0.1 g/L to about 6 g/L, about 0.1 g/L to about 7 g/L, about 0.1 g/L to about 8 g/L, about 0.1 g/L to about 9 g/L, about 0.1 g/L to about 10 g/L, about 0.5 g/L to about 1 g/L, about 0.5 g/L to about 2 g/L, about 0.5 g/L to about 3 g/L, about 0.5 g/L to about 4 g/L, about 0.5 g/L to about 5 g/L, about 0.5 g/L to about 6 g/L, about 0.5 g/L to about 7 g/L, about 0.5 g/L to about 8 g/L, about 0.5 g/L to about 9 g/L, about 0.5 g/L to about 10 g/L, about 1 g/L to about 2 g/L, about 1 g/L to about 3 g/L, about 1 g/L to about 4 g/L, about 1 g/L to about 5 g/L, about 1 g/L to about 6 g/L, about 1 g/L to about 7 g/L, about 1 g/L to about 8 g/L, about 1 g/L to about 9 g/L, about 1 g/L to about 10 g/L, about 2 g/L to about 3 g/L, about 2 g/L to about 4 g/L, about 2 g/L to about 5 g/L, about 2 g/L to about 6 g/L, about 2 g/L to about 7 g/L, about 2 g/L to about 8 g/L, about 2 g/L to about 9 g/L, about 2 g/L to about 10 g/L, about 3 g/L to about 4 g/L, about 3 g/L to about 5 g/L, about 3 g/L to about 6 g/L, about 3 g/L to about 7 g/L, about 3 g/L to about 8 g/L, about 3 g/L to about 9 g/L, about 3 g/L to about 10 g/L, about 4 g/L to about 5 g/L, about 4 g/L to about 6 g/L, about 4 g/L to about 7 g/L, about 4 g/L to about 8 g/L, about 4 g/L to about 9 g/L, about 4 g/L to about 10 g/L, about 5 g/L to about 6 g/L, about 5 g/L to about 7 g/L, about 5 g/L to about 8 g/L, about 5 g/L to about 9 g/L, about 5 g/L to about 10 g/L, about 6 g/L to about 7 g/L, about 6 g/L to about 8 g/L, about 6 g/L to about 9 g/L, about 6 g/L to about 10 g/L, about 7 g/L to about 8 g/L, about 7 g/L to about 9 g/L, about 7 g/L to about 10 g/L, about 8 g/L to about 9 g/L, about 8 g/L to about 10 g/L, or about 9 g/L to about 10 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.1 g/L, about 0.5 g/L, about 1 g/L, about 2 g/L, about 3 g/L, about 4 g/L, about 5 g/L, about 6 g/L, about 7 g/L, about 8 g/L, about 9 g/L, or about 10 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of at least about 0.1 g/L, about 0.5 g/L, about 1 g/L, about 2 g/L, about 3 g/L, about 4 g/L, about 5 g/L, about 6 g/L, about 7 g/L, about 8 g/L, or about 9 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of at most about 0.5 g/L, about 1 g/L, about 2 g/L, about 3 g/L, about 4 g/L, about 5 g/L, about 6 g/L, about 7 g/L, about 8 g/L, about 9 g/L, or about 10 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.2 to about 5 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.2 g/L to about 5 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.2 g/L to about 0.3 g/L, about 0.2 g/L to about 0.4 g/L, about 0.2 g/L to about 0.5 g/L, about 0.2 g/L to about 0.75 g/L, about 0.2 g/L to about 1 g/L, about 0.2 g/L to about 1.25

g/L, about 0.2 g/L to about 1.5 g/L, about 0.2 g/L to about 2 g/L, about 0.2 g/L to about 3 g/L, about 0.2 g/L to about 4 g/L, about 0.2 g/L to about 5 g/L, about 0.3 g/L to about 0.4 g/L, about 0.3 g/L to about 0.5 g/L, about 0.3 g/L to about 0.75 g/L, about 0.3 g/L to about 1 g/L, about 0.3 g/L to about 1.25 g/L, about 0.3 g/L to about 1.5 g/L, about 0.3 g/L to about 2 g/L, about 0.3 g/L to about 3 g/L, about 0.3 g/L to about 4 g/L, about 0.3 g/L to about 5 g/L, about 0.4 g/L to about 0.5 g/L, about 0.4 g/L to about 0.75 g/L, about 0.4 g/L to about 1 g/L, about 0.4 g/L to about 1.25 g/L, about 0.4 g/L to about 1.5 g/L, about 0.4 g/L to about 2 g/L, about 0.4 g/L to about 3 g/L, about 0.4 g/L to about 4 g/L, about 0.4 g/L to about 5 g/L, about 0.5 g/L to about 0.75 g/L, about 0.5 g/L to about 1 g/L, about 0.5 g/L to about 1.25 g/L, about 0.5 g/L to about 1.5 g/L, about 0.5 g/L to about 2 g/L, about 0.5 g/L to about 3 g/L, about 0.5 g/L to about 4 g/L, about 0.5 g/L to about 5 g/L, about 0.75 g/L to about 1 g/L, about 0.75 g/L to about 1.25 g/L, about 0.75 g/L to about 1.5 g/L, about 0.75 g/L to about 2 g/L, about 0.75 g/L to about 3 g/L, about 0.75 g/L to about 4 g/L, about 0.75 g/L to about 5 g/L, about 1 g/L to about 1.25 g/L, about 1 g/L to about 1.5 g/L, about 1 g/L to about 2 g/L, about 1 g/L to about 3 g/L, about 1 g/L to about 4 g/L, about 1 g/L to about 5 g/L, about 1.25 g/L to about 1.5 g/L, about 1.25 g/L to about 2 g/L, about 1.25 g/L to about 3 g/L, about 1.25 g/L to about 4 g/L, about 1.25 g/L to about 5 g/L, about 1.5 g/L to about 2 g/L, about 1.5 g/L to about 3 g/L, about 1.5 g/L to about 4 g/L, about 1.5 g/L to about 5 g/L, about 2 g/L to about 3 g/L, about 2 g/L to about 4 g/L, about 2 g/L to about 5 g/L, about 3 g/L to about 4 g/L, about 3 g/L to about 5 g/L, or about 4 g/L to about 5 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of about 0.2 g/L, about 0.3 g/L, about 0.4 g/L, about 0.5 g/L, about 0.75 g/L, about 1 g/L, about 1.25 g/L, about 1.5 g/L, about 2 g/L, about 3 g/L, about 4 g/L, or about 5 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of at least about 0.2 g/L, about 0.3 g/L, about 0.4 g/L, about 0.5 g/L, about 0.75 g/L, about 1 g/L, about 1.25 g/L, about 1.5 g/L, about 2 g/L, about 3 g/L, or about 4 g/L. In some embodiments, the methods herein are used to obtain a yield of active, soluble, and/or intact recombinant protein of interest of at most about 0.3 g/L, about 0.4 g/L, about 0.5 g/L, about 0.75 g/L, about 1 g/L, about 1.25 g/L, about 1.5 g/L, about 2 g/L, about 3 g/L, about 4 g/L, or about 5 g/L.

[0270] In embodiments, the amount of active, soluble, and/or intact recombinant protein of interest is about 10% to about 100% of the amount of the total active, soluble, and/or intact recombinant protein of interest produced. In embodiments, this amount is about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95% or about 99%, or about 100% of the amount of the active, soluble, and/or intact recombinant

protein of interest produced. In embodiments, this amount is about 10% to about 20%, 20% to about 50%, about 25% to about 50%, about 25% to about 50%, about 25% to about 95%, about 30% to about 50%, about 30% to about 40%, about 30% to about 60%, about 30% to about 70%, about 35% to about 50%, about 35% to about 70%, about 35% to about 75%, about 35% to about 95%, about 40% to about 50%, about 40% to about 95%, about 50% to about 75%, about 50% to about 95%, about 70% to about 95%, or about 80 to about 100% of the amount of the active, soluble, and/or intact recombinant protein of interest produced.

[0271] In some embodiments, the amount of active, soluble, and/or intact recombinant protein of interest is expressed as a percentage of the total active, soluble, and/or intact protein produced in a culture. Data expressed in terms of active, soluble, and/or intact recombinant protein of interest weight/volume of cell culture at a given cell density can be converted to data expressed as percent recombinant protein of total cell protein. It is within the capabilities of a skilled artisan to convert volumetric protein yield to % total cell protein, for example, knowing the amount of total cell protein per volume of cell culture at the given cell density. This number can be determined if one knows 1) the cell weight/volume of culture at the given cell density, and 2) the percent of cell weight comprised by total protein. For example, at an OD₅₅₀ of 1.0, the dry cell weight of *E. coli* is reported to be 0.5 grams/liter (“Production of Heterologous Proteins from Recombinant DNA Escherichia coli in Bench Fermentors,” Lin, N.S., and Swartz, J.R., 1992, METHODS: A Companion to Methods in Enzymology 4: 159-168). A bacterial cell is comprised of polysaccharides, lipids, and nucleic acids, as well as proteins. An *E. coli* cell is reported to be about 52.4 to 55% protein by references including, but not limited to, Da Silva, N.A., et al., 1986, “Theoretical Growth Yield Estimates for Recombinant Cells,” Biotechnology and Bioengineering, Vol. XXVIII: 741-746, estimating protein to make up 52.4% by weight of *E. coli* cells, and “Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology,” 1987, Ed. in Chief Frederick C. Neidhardt, Vol. 1, pp. 3-6, reporting protein content in *E. coli* as 55% dry cell weight. Using the measurements above (i.e., a dry cell weight of 0.5 grams/liter, and protein as 55% cell weight), the amount of total cell protein per volume of cell culture at an A₅₅₀ of 1.0 for *E. coli* is calculated as 275 µg total cell protein/ml/A₅₅₀. A calculation of total cell protein per volume of cell culture based on wet cell weight can use, e.g., the determination by Glazyrina, et al. (Microbial Cell Factories 2010, 9:42, incorporated herein by reference) that an A₆₀₀ of 1.0 for *E. coli* resulted in a wet cell weight of 1.7 grams/liter and a dry cell weight of 0.39 grams/liter. For example, using this wet cell weight to dry cell weight comparison, and protein as 55% dry cell weight as described above, the amount of total cell protein per volume of cell culture at an A₆₀₀ of 1.0 for *E. coli* can be calculated as 215 µg total cell protein/ml/A₆₀₀. For *Pseudomonas fluorescens*, the amount of total cell protein per volume

of cell culture at a given cell density is similar to that found for *E. coli*. *P. fluorescens*, like *E. coli*, is a gram-negative, rod-shaped bacterium. The dry cell weight of *P. fluorescens* ATCC 11150 as reported by Edwards, et al., 1972, "Continuous Culture of *Pseudomonas fluorescens* with Sodium Maleate as a Carbon Source," Biotechnology and Bioengineering, Vol. XIV, pages 123-147, is 0.5 grams/liter/A500. This is the same weight reported by Lin, et al., for *E. coli* at an A550 of 1.0. Light scattering measurements made at 500nm and at 550nm are expected to be very similar. The percent of cell weight comprised by total cell protein for *P. fluorescens* HK44 is described as 55% by, e.g., Yarwood, et al., July 2002, "Noninvasive Quantitative Measurement of Bacterial Growth in Porous Media under Unsaturated-Flow Conditions," Applied and Environmental Microbiology 68(7):3597-3605. This percentage is similar to or the same as those given for *E. coli* by the references described above.

[0272] In embodiments, the amount of active, soluble, and/or intact recombinant protein of interest produced is about 0.1% to about 95% of the total active, soluble, and/or intact protein produced in a culture. In embodiments, this amount is more than about 0.1%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the total active, soluble, and/or intact protein produced in a culture. In embodiments, this amount is about 0.1%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the total active, soluble, and/or intact protein produced in a culture. In embodiments, this amount is about 5% to about 95%, about 10% to about 85%, about 20% to about 75%, about 30% to about 65%, about 40% to about 55%, about 1% to about 95%, about 5% to about 30%, about 1% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50 to about 60%, about 60% to about 70%, or about 80% to about 90% of the total active, soluble, and/or intact protein produced in a culture.

[0273] In embodiments, the amount of active, soluble, and/or intact recombinant protein of interest produced is about 0.1% to about 50% of the dry cell weight (DCW). In embodiments, this amount is more than about 0.1%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, or 50% of DCW. In embodiments, this amount is about 0.1%, 0.5%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, or 50% of DCW. In embodiments, this amount is about 5% to about 50%, about 10% to about 40%, about 20% to about 30%, about 1% to about 20%, about 5% to about 25%, about 1% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, or about 40% to about 50% of the total active, soluble, and/or intact protein produced in a culture.

[0274] In embodiments, the amount of an active, soluble, and/or intact recombinant protein of interest produced using the methods of the invention is greater than the amount of the protein

produced by a control host cell under substantially similar conditions, e.g., the same growth conditions. A control host cell may be a host cell that is the same in all respects to the recombinant gram-negative host cell, but that (a) is not deficient in one or more activities deficient in the recombinant gram-negative host cell, (b) does not overexpress one or more chaperones, folding modulators, or inactivated proteases that are overexpressed in the recombinant gram-negative host cell, or (c) any combination of (a) and (b). A control host cell may be a host cell that has the wild-type background of the recombinant gram-negative host cell, but that (a) is not deficient in one or more activities deficient in the recombinant gram-negative host cell, (b) does not overexpress one or more chaperones, folding modulators, or inactivated proteases that are overexpressed in the recombinant gram-negative host cell, or (c) any combination of (a) and (b). In some embodiments, an active, soluble, and/or intact recombinant protein of interest produced according to the present methods using a recombinant gram-negative host cell of the invention, is produced in an amount greater than the amount of the protein produced by a control host cell. In some embodiments, an active, soluble, and/or intact recombinant protein of interest produced by a recombinant gram-negative host cell of the invention is produced at a yield that is about 1.5 fold to about 10 fold. In some embodiments, an active, soluble, and/or intact recombinant protein of interest produced by a recombinant gram-negative host cell of the invention is produced at a yield that is about 1.5 fold to about 2 fold, about 1.5 fold to about 2.5 fold, about 1.5 fold to about 3 fold, about 1.5 fold to about 3.5 fold, about 1.5 fold to about 4 fold, about 1.5 fold to about 5 fold, about 1.5 fold to about 6 fold, about 1.5 fold to about 7 fold, about 1.5 fold to about 8 fold, about 1.5 fold to about 9 fold, about 1.5 fold to about 10 fold, about 2 fold to about 2.5 fold, about 2 fold to about 3 fold, about 2 fold to about 3.5 fold, about 2 fold to about 4 fold, about 2 fold to about 5 fold, about 2 fold to about 6 fold, about 2 fold to about 7 fold, about 2 fold to about 8 fold, about 2 fold to about 9 fold, about 2 fold to about 10 fold, about 2.5 fold to about 3 fold, about 2.5 fold to about 3.5 fold, about 2.5 fold to about 4 fold, about 2.5 fold to about 5 fold, about 2.5 fold to about 6 fold, about 2.5 fold to about 7 fold, about 2.5 fold to about 8 fold, about 2.5 fold to about 9 fold, about 2.5 fold to about 10 fold, about 3 fold to about 3.5 fold, about 3 fold to about 4 fold, about 3 fold to about 5 fold, about 3 fold to about 6 fold, about 3 fold to about 7 fold, about 3 fold to about 8 fold, about 3 fold to about 9 fold, about 3 fold to about 10 fold, about 3.5 fold to about 4 fold, about 3.5 fold to about 5 fold, about 3.5 fold to about 6 fold, about 3.5 fold to about 7 fold, about 3.5 fold to about 8 fold, about 3.5 fold to about 9 fold, about 3.5 fold to about 10 fold, about 4 fold to about 5 fold, about 4 fold to about 6 fold, about 4 fold to about 7 fold, about 4 fold to about 8 fold, about 4 fold to about 9 fold, about 4 fold to about 10 fold, about 5 fold to about 6 fold, about 5 fold to about 7 fold, about 5 fold to about 8 fold, about 5 fold to about 9 fold, about 5 fold to

about 10 fold, about 6 fold to about 7 fold, about 6 fold to about 8 fold, about 6 fold to about 9 fold, about 6 fold to about 10 fold, about 7 fold to about 8 fold, about 7 fold to about 9 fold, about 7 fold to about 10 fold, about 8 fold to about 9 fold, about 8 fold to about 10 fold, or about 9 fold to about 10 fold greater than the amount of the protein produced by a control host cell. In some embodiments, an active, soluble, and/or intact recombinant protein of interest produced by a recombinant gram-negative host cell of the invention is produced at a yield that is about 1.5 fold, about 2 fold, about 2.5 fold, about 3 fold, about 3.5 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, or about 10 fold greater than the amount of the protein produced by a control host cell. In some embodiments, active, soluble, and/or intact recombinant protein of interest produced by a recombinant gram-negative host cell of the invention is produced at a yield that is at least about 1.5 fold, about 2 fold, about 2.5 fold, about 3 fold, about 3.5 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, or about 9 fold greater than the amount of the protein produced by a control host cell. In some embodiments, active, soluble, and/or intact recombinant protein of interest produced by a recombinant gram-negative host cell of the invention is produced at a yield that is at most about 2 fold, about 2.5 fold, about 3 fold, about 3.5 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, or about 10 fold greater than the amount of the protein produced by a control host cell.

Activity assays

[0275] Assays for evaluating the activity of a recombinant protein of interest are known in the art and include but are not limited to fluorometric, colorimetric, chemiluminescent, spectrophotometric, and other enzyme assays available to one of skill in the art. A binding protein such as an antibody, antibody fragment, or derivative thereof may be evaluated by any appropriate target binding assay known in the art. These assays may be used to compare activity of a preparation of a recombinant protein of interest to a commercial or other preparation of the recombinant protein.

[0276] In embodiments, activity is represented by the percent active protein in the extract supernatant as compared with the total amount assayed. This is based on the amount of protein determined to be active by the assay relative to the total amount of protein used in assay. In other embodiments, activity is represented by the % activity level of the protein compared to a standard, e.g., native protein. This is based on the amount of active protein in supernatant extract sample relative to the amount of active protein in a standard sample (where the same amount of protein from each sample is used in assay).

[0277] In embodiments, about 40% to about 100% of the peptide, polypeptide or protein of interest, is determined to be active. In embodiments, about 40%, about 50%, about 60%, about

70%, about 80%, about 90%, or about 100% of the peptide, polypeptide or protein of interest is determined to be active. In embodiments, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 80% to about 90%, about 90% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 40% to about 90%, about 40% to about 95%, about 50% to about 90%, about 50% to about 95%, about 50% to about 100%, about 60% to about 90%, about 60% to about 95%, about 60% to about 100%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, or about 70% to about 100% of the peptide, polypeptide or protein of interest is determined to be active.

[0278] In other embodiments, about 75% to about 100% of the peptide, polypeptide or protein of interest is determined to be active. In embodiments, about 75% to about 80%, about 75% to about 85%, about 75% to about 90%, about 75% to about 95%, about 80% to about 85%, about 80% to about 90%, about 80% to about 95%, about 80% to about 100%, about 85% to about 90%, about 85% to about 95%, about 85% to about 100%, about 90% to about 95%, about 90% to about 100%, or about 95% to about 100% of the peptide, polypeptide or protein of interest is determined to be active.

[0279] SImT Secretion Signal Peptide

[0280] Compositions and methods for producing high levels of properly processed recombinant proteins or polypeptides in a host cell are provided. In some aspects, a novel secretion signal, SImt, that promotes the targeting of the recombinant protein or polypeptide of interest to the periplasm of Gram-negative bacteria or into the extracellular environment is provided. The SImt periplasmic secretion signal peptide disclosed herein enables transport of proteins across the inner membrane to the periplasmic space in Gram negative bacteria. In some aspects, SImt periplasmic secretion signal peptide provided herein promotes the targeting of the recombinant protein or polypeptide of interest to the extracellular space in Gram-positive bacteria. Periplasmic protein expression allows for proper formation of disulfide bonds in the periplasm and can result in high level recombinant protein expression. Expression to the periplasmic space may enable more efficient recovery / purification of the recombinant protein. For the purposes of the present disclosure, a “secretion signal,” “secretion leader,” “secretion signal polypeptide,” “signal peptide,” “leader peptide” or “leader sequence” are intended to refer to a peptide sequence (or the polynucleotide encoding the peptide sequence) that is useful for targeting a protein or polypeptide of interest to a cell compartment, e.g., the periplasm of Gram-negative bacteria or into the extracellular space. The secretion signal sequence includes the SImt secretion signal (amino acid sequence set forth in SEQ ID NO: 11), and fragments and variants thereof. One example of a nucleotide sequence encoding SEQ ID NO: 11 and useful in the present

methods is provided in SEQ ID NO: 12. As known to those of skill in the art, an amino acid sequence can be encoded by different nucleotide sequences due to the redundancy in the genetic code. The compositions and methods of the present invention thus may include the same secretion signal amino acid sequence whilst encoded by different nucleotide sequences. Also provided herein are fragments and variants of the secretion signal peptide sequence that can direct periplasmic expression of an operably linked recombinant protein or polypeptide of interest.

[0281] A secretion signal coding sequence that encodes the amino acid sequence as set forth in SEQ ID NO: 11 may be fused to the N-terminus of a sequence encoding a heterologous recombinant protein or polypeptide of interest to be expressed and targeted to the host cell periplasm or into the extracellular space. As used herein with regard to a heterologous secretion signal and protein or polypeptide of interest, a “heterologous” secretion signal peptide is not native to the protein or polypeptide of interest. Conversely, with regard to a secretion signal peptide, a “heterologous” protein or polypeptide of interest is not native to the secretion signal. With regard to SEQ ID NO: 11, a heterologous protein or polypeptide of interest is one that is not *P. fluorescens* Soluble lytic murein transglycosylase (SlmT). With regard to a construct comprising a secretion signal coding sequence that encodes the amino acid sequence as set forth in SEQ ID NO: 11, a sequence encoding a heterologous protein or polypeptide of interest is one that does not encode *P. fluorescens* Soluble lytic murein transglycosylase (SlmT). In the context of the host cell, the term heterologous may refer to a protein or polypeptide of interest that is not native to a particular host cell.

[0282] The invention includes a method of producing a protein or polypeptide of interest in a prokaryotic host cell, comprising producing the protein or polypeptide of interest in the periplasm of a prokaryotic host cell cultured in a cell culture growth medium, wherein the prokaryotic host cell comprises an expression construct comprising a nucleic acid encoding a recombinant polypeptide comprising the protein or polypeptide of interest operably linked to a secretion signal peptide that directs expression of the protein or polypeptide of interest to the periplasm of the prokaryotic host cell, wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 11, and wherein the secretion signal peptide is not native to the protein or polypeptide of interest.

[0283] In some embodiments, the protein or polypeptide of interest is expressed in the periplasm properly cleaved from the secretion signal peptide, e.g., SEQ ID NO: 11. In some embodiments, the secretion signal peptide directs expression of the protein or polypeptide of interest to the periplasm or the extracellular space of a prokaryotic host cell in properly cleaved form, soluble form, active form, or any combination thereof. A correctly or properly cleaved or processed

protein or polypeptide of interest may have an intact or substantially intact N-terminus. In some embodiments, the properly cleaved protein or polypeptide of interest having an intact or substantially intact N-terminus comprises the N-terminal methionine. In some embodiments, the properly cleaved protein or polypeptide of interest having an intact or substantially intact N-terminus does not comprise the N-terminal methionine. A protein or polypeptide of interest may require a substantially intact N-terminus for activity, solubility, or both. In some embodiments, a protein or polypeptide of interest has about 80-100% activity when compared to a control. In some embodiments, the control is the same protein or polypeptide of interest that comprises an N-terminal methionine. In some embodiments, the control is the same protein or polypeptide of interest that does not comprise an N-terminal methionine. In some embodiments, the control is the same protein or polypeptide of interest that has a substantially intact N-terminus. In some embodiments, the expressed or produced protein or polypeptide of interest has an activity relative to a control of about 80% to about 100%. In some embodiments, a protein or polypeptide of interest having a substantially intact N-terminus has an activity relative to a control of about 80% to about 85%, about 80% to about 90%, about 80% to about 92%, about 80% to about 94%, about 80% to about 95%, about 80% to about 96%, about 80% to about 97%, about 80% to about 98%, about 80% to about 99%, about 80% to about 100%, about 85% to about 90%, about 85% to about 92%, about 85% to about 94%, about 85% to about 95%, about 85% to about 96%, about 85% to about 97%, about 85% to about 98%, about 85% to about 99%, about 85% to about 100%, about 90% to about 92%, about 90% to about 94%, about 90% to about 95%, about 90% to about 96%, about 90% to about 97%, about 90% to about 98%, about 90% to about 99%, about 90% to about 100%, about 92% to about 94%, about 92% to about 95%, about 92% to about 96%, about 92% to about 97%, about 92% to about 98%, about 92% to about 99%, about 92% to about 100%, about 94% to about 95%, about 94% to about 96%, about 94% to about 97%, about 94% to about 98%, about 94% to about 99%, about 94% to about 100%, about 95% to about 96%, about 95% to about 97%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 97%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98% to about 100%, or about 99% to about 100%. In some embodiments, a protein or polypeptide of interest having a substantially intact N-terminus has an activity relative to a control of about 80%, about 85%, about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100%. In some embodiments, a protein or polypeptide of interest having a substantially intact N-terminus has an activity relative to a control of at least about 80%, about 85%, about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%. In some embodiments, a

protein or polypeptide of interest having a substantially intact N-terminus has an activity relative to a control of at most about 85%, about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100%.

[0284] In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L to about 50 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L to about 3 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L to about 0.2 g/L, about 0.1 g/L to about 0.3 g/L, about 0.1 g/L to about 0.4 g/L, about 0.1 g/L to about 0.5 g/L, about 0.1 g/L to about 0.6 g/L, about 0.1 g/L to about 0.7 g/L, about 0.1 g/L to about 0.8 g/L, about 0.1 g/L to about 0.9 g/L, about 0.1 g/L to about 1 g/L, about 0.1 g/L to about 2 g/L, about 0.1 g/L to about 3 g/L, about 0.2 g/L to about 0.3 g/L, about 0.2 g/L to about 0.4 g/L, about 0.2 g/L to about 0.5 g/L, about 0.2 g/L to about 0.6 g/L, about 0.2 g/L to about 0.7 g/L, about 0.2 g/L to about 0.8 g/L, about 0.2 g/L to about 0.9 g/L, about 0.2 g/L to about 1 g/L, about 0.2 g/L to about 2 g/L, about 0.2 g/L to about 3 g/L, about 0.3 g/L to about 0.4 g/L, about 0.3 g/L to about 0.5 g/L, about 0.3 g/L to about 0.6 g/L, about 0.3 g/L to about 0.7 g/L, about 0.3 g/L to about 0.8 g/L, about 0.3 g/L to about 0.9 g/L, about 0.3 g/L to about 1 g/L, about 0.3 g/L to about 2 g/L, about 0.3 g/L to about 3 g/L, about 0.4 g/L to about 0.5 g/L, about 0.4 g/L to about 0.6 g/L, about 0.4 g/L to about 0.7 g/L, about 0.4 g/L to about 0.8 g/L, about 0.4 g/L to about 0.9 g/L, about 0.4 g/L to about 1 g/L, about 0.4 g/L to about 2 g/L, about 0.4 g/L to about 3 g/L, about 0.5 g/L to about 0.6 g/L, about 0.5 g/L to about 0.7 g/L, about 0.5 g/L to about 0.8 g/L, about 0.5 g/L to about 0.9 g/L, about 0.5 g/L to about 1 g/L, about 0.5 g/L to about 2 g/L, about 0.5 g/L to about 3 g/L, about 0.6 g/L to about 0.7 g/L, about 0.6 g/L to about 0.8 g/L, about 0.6 g/L to about 0.9 g/L, about 0.6 g/L to about 1 g/L, about 0.6 g/L to about 2 g/L, about 0.6 g/L to about 3 g/L, about 0.7 g/L to about 0.8 g/L, about 0.7 g/L to about 0.9 g/L, about 0.7 g/L to about 1 g/L, about 0.7 g/L to about 2 g/L, about 0.7 g/L to about 3 g/L, about 0.8 g/L to about 0.9 g/L, about 0.8 g/L to about 1 g/L, about 0.8 g/L to about 2 g/L, about 0.8 g/L to about 3 g/L, about 0.9 g/L to about 1 g/L, about 0.9 g/L to about 2 g/L, about 0.9 g/L to about 3 g/L, about 1 g/L to about 2 g/L, about 1 g/L to about 3 g/L, or about 2 g/L to about 3 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L, about 0.2 g/L, about 0.3 g/L, about 0.4 g/L, about 0.5 g/L, about 0.6 g/L, about 0.7 g/L, about 0.8 g/L, about 0.9 g/L, about 1 g/L, about 2 g/L, or about 3 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at at least about 0.1 g/L, about 0.2 g/L, about 0.3 g/L, about 0.4 g/L, about 0.5 g/L, about 0.6 g/L, about 0.7 g/L, about 0.8 g/L, about 0.9 g/L, about 1 g/L, or about 2 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at at most about 0.2 g/L, about

0.3 g/L, about 0.4 g/L, about 0.5 g/L, about 0.6 g/L, about 0.7 g/L, about 0.8 g/L, about 0.9 g/L, about 1 g/L, about 2 g/L, or about 3 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L to about 50 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L to about 0.5 g/L, about 0.1 g/L to about 1 g/L, about 0.1 g/L to about 2 g/L, about 0.1 g/L to about 5 g/L, about 0.1 g/L to about 10 g/L, about 0.1 g/L to about 15 g/L, about 0.1 g/L to about 20 g/L, about 0.1 g/L to about 25 g/L, about 0.1 g/L to about 30 g/L, about 0.1 g/L to about 40 g/L, about 0.1 g/L to about 50 g/L, about 0.5 g/L to about 1 g/L, about 0.5 g/L to about 2 g/L, about 0.5 g/L to about 5 g/L, about 0.5 g/L to about 10 g/L, about 0.5 g/L to about 15 g/L, about 0.5 g/L to about 20 g/L, about 0.5 g/L to about 25 g/L, about 0.5 g/L to about 30 g/L, about 0.5 g/L to about 40 g/L, about 0.5 g/L to about 50 g/L, about 1 g/L to about 2 g/L, about 1 g/L to about 5 g/L, about 1 g/L to about 10 g/L, about 1 g/L to about 15 g/L, about 1 g/L to about 20 g/L, about 1 g/L to about 25 g/L, about 1 g/L to about 30 g/L, about 1 g/L to about 40 g/L, about 1 g/L to about 50 g/L, about 2 g/L to about 5 g/L, about 2 g/L to about 10 g/L, about 2 g/L to about 15 g/L, about 2 g/L to about 20 g/L, about 2 g/L to about 25 g/L, about 2 g/L to about 30 g/L, about 2 g/L to about 40 g/L, about 2 g/L to about 50 g/L, about 5 g/L to about 10 g/L, about 5 g/L to about 15 g/L, about 5 g/L to about 20 g/L, about 5 g/L to about 25 g/L, about 5 g/L to about 30 g/L, about 5 g/L to about 40 g/L, about 5 g/L to about 50 g/L, about 10 g/L to about 15 g/L, about 10 g/L to about 20 g/L, about 10 g/L to about 25 g/L, about 10 g/L to about 30 g/L, about 10 g/L to about 40 g/L, about 10 g/L to about 50 g/L, about 15 g/L to about 20 g/L, about 15 g/L to about 25 g/L, about 15 g/L to about 30 g/L, about 15 g/L to about 40 g/L, about 15 g/L to about 50 g/L, about 20 g/L to about 25 g/L, about 20 g/L to about 30 g/L, about 20 g/L to about 40 g/L, about 20 g/L to about 50 g/L, about 25 g/L to about 30 g/L, about 25 g/L to about 40 g/L, about 25 g/L to about 50 g/L, about 30 g/L to about 40 g/L, about 30 g/L to about 50 g/L, or about 40 g/L to about 50 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at about 0.1 g/L, about 0.5 g/L, about 1 g/L, about 2 g/L, about 5 g/L, about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 40 g/L, or about 50 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at at least about 0.1 g/L, about 0.5 g/L, about 1 g/L, about 2 g/L, about 5 g/L, about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, or about 40 g/L. In some embodiments, the process produces correctly processed periplasmic or extracellular protein at at most about 0.5 g/L, about 1 g/L, about 2 g/L, about 5 g/L, about 10 g/L, about 15 g/L, about 20 g/L, about 25 g/L, about 30 g/L, about 40 g/L, or about 50 g/L.

[0285] In some embodiments, the % of total recombinant protein or polypeptide that is produced in correctly processed form is about 5 to about 100. In some embodiments, the % of total

recombinant protein or polypeptide that is produced in correctly processed form is about 5 to about 10, about 5 to about 20, about 5 to about 30, about 5 to about 40, about 5 to about 50, about 5 to about 60, about 5 to about 70, about 5 to about 80, about 5 to about 90, about 5 to about 95, about 5 to about 100, about 10 to about 20, about 10 to about 30, about 10 to about 40, about 10 to about 50, about 10 to about 60, about 10 to about 70, about 10 to about 80, about 10 to about 90, about 10 to about 95, about 10 to about 100, about 20 to about 30, about 20 to about 40, about 20 to about 50, about 20 to about 60, about 20 to about 70, about 20 to about 80, about 20 to about 90, about 20 to about 95, about 20 to about 100, about 30 to about 40, about 30 to about 50, about 30 to about 60, about 30 to about 70, about 30 to about 80, about 30 to about 90, about 30 to about 95, about 30 to about 100, about 40 to about 50, about 40 to about 60, about 40 to about 70, about 40 to about 80, about 40 to about 90, about 40 to about 95, about 40 to about 100, about 50 to about 60, about 50 to about 70, about 50 to about 80, about 50 to about 90, about 50 to about 95, about 50 to about 100, about 60 to about 70, about 60 to about 80, about 60 to about 90, about 60 to about 95, about 60 to about 100, about 70 to about 80, about 70 to about 90, about 70 to about 95, about 70 to about 100, about 80 to about 90, about 80 to about 95, about 80 to about 100, about 90 to about 95, about 90 to about 100, or about 95 to about 100. In some embodiments, the % of total recombinant protein or polypeptide that is produced in correctly processed form is about 5, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 95, or about 100. In some embodiments, the % of total recombinant protein or polypeptide that is produced in correctly processed form is at least about 5, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, or about 95. In some embodiments, the % of total recombinant protein or polypeptide that is produced in correctly processed form is at most about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 95, or about 100.

[0286] The following patents and patent applications are incorporated herein by reference in their entirety, including as they relate to processing/cleavage and periplasmic expression of recombinant proteins and polypeptides fused to secretion signal peptides: U.S. Pat. No. 7,618,799, “Bacterial leader sequences for increased expression,” in U.S. Pat. No. 7,985,564, “Expression systems with Sec-system secretion,” in U.S. Pat. Nos. 9,394,571 and 9,580,719, both titled “Method for Rapidly Screening Microbial Hosts to Identify Certain Strains with Improved Yield and/or Quality in the Expression of Heterologous Proteins,” U.S. Pat. No. 9,453,251, “Expression of Mammalian Proteins in *Pseudomonas fluorescens*,” U.S. Pat. No. 8,603,824, “Process for Improved Protein Expression by Strain Engineering,” and U.S. Pat. No. 8,530,171, “High Level Expression of Recombinant Toxin Proteins,” and U.S. Pat. Pub. No. 2019/0127744, “Bacterial Leader Sequences for Periplasmic Protein Expression.”

[0287] In embodiments, the secretion signal sequence is identical to or substantially identical to a secretion signal peptide set forth in SEQ ID NO: 11, and/or is encoded by a polynucleotide sequence set forth in SEQ ID NO: 12. In another embodiment, the secretion signal sequence comprises at least amino acids 2-29 of SEQ ID NO: 11. In yet another embodiment, the secretion signal sequence comprises a fragment of SEQ ID NO: 11, which is truncated by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids from the amino terminus but retains biological activity, i.e., secretion signal activity.

[0288] In one embodiment the amino acid sequence of the peptide is a variant of a given original peptide, wherein the sequence of the variant is obtainable by replacing up to or about 30% of the original peptide's amino acid residues with other amino acid residue(s), including up to about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, or 30%, provided that the variant retains the desired function of the original peptide. A variant amino acid with substantial homology will be at least about 70%, at least about 75%, at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or at least about 99% homologous to the original peptide. A variant amino acid sequence may be obtained in various ways including amino acid substitutions, deletions, truncations, and insertions of one or more amino acids of SEQ ID NO: 11. In some embodiments, a variant amino acid sequence comprises 1-9 amino acid substitutions, deletions, insertions, or any combination thereof. In some embodiments, the number of amino acid substitutions, deletions, insertions, or any combination thereof, in a variant of SEQ ID NO: 11, is 1 to 10. In some embodiments, the number of amino acid substitutions, deletions, insertions, or any combination thereof, in a variant of SEQ ID NO: 11, is 1 to 2, 1 to 3, 1 to 4, 1 to 5, 1 to 6, 1 to 7, 1 to 8, 1 to 9, 1 to 10, 2 to 3, 2 to 4, 2 to 5, 2 to 6, 2 to 7, 2 to 8, 2 to 9, 2 to 10, 3 to 4, 3 to 5, 3 to 6, 3 to 7, 3 to 8, 3 to 9, 3 to 10, 4 to 5, 4 to 6, 4 to 7, 4 to 8, 4 to 9, 4 to 10, 5 to 6, 5 to 7, 5 to 8, 5 to 9, 5 to 10, 6 to 7, 6 to 8, 6 to 9, 6 to 10, 7 to 8, 7 to 9, 7 to 10, 8 to 9, 8 to 10, or 9 to 10. In some embodiments, the number of amino acid substitutions, deletions, insertions, or any combination thereof, in a variant of SEQ ID NO: 11, is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the number of amino acid substitutions, deletions, insertions, or any combination thereof, in a variant of SEQ ID NO: 11, is at least 1, 2, 3, 4, 5, 6, 7, 8, or 9. In some embodiments, the number of amino acid substitutions, deletions, insertions, or any combination thereof, in a variant of SEQ ID NO: 11, is at most 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[0289] By “substantially homologous,” “substantially identical,” or “substantially similar” is intended an amino acid or nucleotide sequence that has about or at least about 60%, about or at least about 65%, about or at least about 70%, about or at least about 75%, about or at least about 80%, about or at least about 85%, about or at least about 81%, about or at least about 82%, about

or at least about 83%, about or at least about 84%, about or at least about 85%, about or at least about 86%, about or at least about 87%, about or at least about 88%, about or at least about 89%, about or at least about 90%, about or at least about 91%, about or at least about 92%, about or at least about 93%, about or at least about 94%, about or at least about 95%, about or at least about 96%, about or at least about 97%, about or at least about 98% or about or at least about 99%, or greater sequence identity as compared to a reference sequence using a suitable alignment program described herein or known in the art using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

[0290] In embodiments, a secretion signal peptide used in the present invention may include one or more modifications of a “non-essential” amino acid residue. In this context, a “non-essential” amino acid residue is a residue that can be altered, e.g., deleted, substituted, or derivatized, in the novel amino acid sequence without abolishing or substantially reducing the activity (e.g., the agonist activity) of the original secretion signal peptide (also referred to as the “analog” or “reference” peptide). In some embodiments, a secretion signal peptide may include one or more modifications of an “essential” amino acid residue. In this context, an “essential” amino acid residue is a residue that when altered, e.g., deleted, substituted, or derivatized, in the novel amino acid sequence the activity of the reference peptide is substantially reduced or abolished. In such embodiments where an essential amino acid residue is altered, the modified secretion signal peptide may possess an activity of the original secretion signal. The substitutions, insertions and deletions may be at the N-terminal or C-terminal end, or may be at internal portions of the secretion signal. By way of example, the secretion signal peptide may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more substitutions, both in a consecutive manner or spaced throughout the secretion signal peptide. Alone or in combination with the substitutions, the secretion signal peptide may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertions, again either in consecutive manner or spaced throughout the secretion signal peptide. The secretion signal peptide, alone or in combination with the substitutions and/or insertions, may also include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more deletions, again either in consecutive manner or spaced throughout the peptide. The secretion signal peptide, alone or in combination with the substitutions, insertions and/or deletions, may also include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more amino acid additions.

[0291] Substitutions include conservative amino acid substitutions. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain, or physicochemical characteristics (e.g., electrostatic, hydrogen bonding, isosteric, hydrophobic features). The amino acids may be naturally occurring or

unnatural. Families of amino acid residues having similar side chains are known in the art. These families include amino acids with basic side chains (e.g. lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, methionine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan), β -branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Substitutions may also include non-conservative changes.

[0292] Variant proteins or polypeptide of interest encompassed herein are biologically active, that is they continue to possess the desired biological activity of the original protein or polypeptide of interest; for example, a variant secretion leader peptide retains secretion signal activity. By “retains activity” is intended that the variant will have about or at least about 30%, about or at least about 35%, about or at least about 40%, about or at least about 45%, about or at least about 50%, about or at least about 55%, about or at least about 60%, about or at least about 65%, about or at least about 70%, about or at least about 75%, about or at least about 80%, about or at least about 85%, about or at least about 81%, about or at least about 82%, about or at least about 83%, about or at least about 84%, about or at least about 85%, about or at least about 86%, about or at least about 87%, about or at least about 88%, about or at least about 89%, about or at least about 90%, about or at least about 91%, about or at least about 92%, about or at least about 93%, about or at least about 94%, about or at least about 95%, about or at least about 96%, about or at least about 97%, about or at least about 98% or about or at least about 99%, about or at least about 100%, about or at least about 110%, about or at least about 125%, about or at least about 150%, about or at least about 200% or greater activity, e.g., secretion signal activity, of the original peptide, protein, or polypeptide.

[0293] Polynucleotides

[0294] The disclosure also includes a nucleic acid with a sequence that encodes a novel secretion signal useful for targeting a protein or polypeptide of interest to the periplasm of Gram-negative bacteria or into the extracellular space. In one embodiment, the isolated polynucleotide encodes a peptide sequence substantially homologous to a Slmt secretion signal peptide. In another embodiment, the present disclosure provides a nucleic acid that encodes a peptide sequence having substantial sequence identity to at least amino acids 2-29 of SEQ ID NO: 11, or provides a nucleic acid having substantial sequence identity to a nucleotide sequence set forth as SEQ ID NO: 12, including biologically active variants and fragments thereof. In another embodiment, the nucleic acid sequence has about or at least about 60%, about or at least about 65%, about or at least about 70%, about or at least about 75%, about or at least about 80%, about or at least about 85%, about or at least about 81%, about or at least about 82%, about or at least about 83%,

about or at least about 84%, about or at least about 85%, about or at least about 86%, about or at least about 87%, about or at least about 88%, about or at least about 89%, about or at least about 90%, about or at least about 91%, about or at least about 92%, about or at least about 93%, about or at least about 94%, about or at least about 95%, about or at least about 96%, about or at least about 97%, about or at least about 98% or about or at least about 99%, or greater identity to a nucleic acid sequence set forth as SEQ ID NO: 12.

[0295] In embodiments, secretion signal peptides herein are encoded by a nucleotide sequence substantially identical to a nucleotide sequence set forth as SEQ ID NO: 12. Corresponding secretion signal peptide sequences having substantial identity to the secretion signal sequences of the present invention can be identified using any appropriate method known in the art, e.g., PCR, hybridization methods, or as described in the literature. See, for example, Sambrook J., and Russell, D.W., 2001, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; and Innis, et al., 1990, *PCR Protocols: A Guide to Methods and Applications*; Academic Press, NY. A variant nucleotide sequence can include a synthetically derived nucleotide sequence that has been generated, for example, by using site-directed mutagenesis. In embodiments, a mutagenized sequence still encodes the secretion signal peptides disclosed herein. Variant secretion signal peptides are biologically active, that is, they continue to possess the desired biological activity of the native protein, that is, they retain secretion signaling activity. By “retains activity” is meant that the variant will have about 30%, about or at least about 35%, about or at least about 40%, about or at least about 45%, about or at least about 50%, about or at least about 55%, about or at least about 60%, about or at least about 65%, about or at least about 70%, about or at least about 75%, about or at least about 80%, about or at least about 85%, about or at least about 81%, about or at least about 82%, about or at least about 83%, about or at least about 84%, about or at least about 85%, about or at least about 86%, about or at least about 87%, about or at least about 88%, about or at least about 89%, about or at least about 90%, about or at least about 91%, about or at least about 92%, about or at least about 93%, about or at least about 94%, about or at least about 95%, about or at least about 96%, about or at least about 97%, about or at least about 98% or about or at least about 99%, about or at least about 100%, about or at least about 110%, about or at least about 125%, about or at least about 150%, about or at least about 200% or greater of the activity of the original secretion signal peptide. Any appropriate method may be used for measuring peptide, protein, or polypeptide activity, e.g., secretion signal activity. Such methods are well known in the art, with examples discussed herein.

[0296] The skilled artisan will further appreciate that changes, in some cases, are introduced by mutation into the nucleotide sequences provided herein thereby leading to changes in the amino

acid sequence of the encoded secretion signal peptides, without altering the biological activity of the secretion signal peptides. Thus, variant isolated nucleic acid molecules are often created by introducing one or more nucleotide substitutions, additions, or deletions into the corresponding nucleotide sequence disclosed herein, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by any standard technique known to those of skill in the art, e.g., site-directed mutagenesis and PCR-mediated mutagenesis.

[0297] Nucleic acid and amino acid sequence identity or homology may be determined according to any suitable method known in the art, including but not limited to those described herein.

[0298] Methods herein, in some cases, comprise expressing polypeptides comprising a protein or polypeptide of interest operably linked to a secretion signal peptide selected from the group consisting of an Slmt secretion signal sequence, or a sequence that is substantially homologous or similar to the secretion signal peptide sequence disclosed herein as SEQ ID NO: 11. In embodiments, the secretion signal peptide sequence is encoded by a nucleotide sequence set forth as SEQ ID NO: 12. In some embodiments, an expression construct is in a *Pseudomonad* host cell. The expression construct, in some cases, is a plasmid. In some embodiments, a plasmid encoding the polypeptide or protein of interest sequence comprises a selection marker, and host cells maintaining the plasmid are grown under selective conditions. In some embodiments, the plasmid does not comprise a selection marker. In some embodiments, the expression construct is integrated into the host cell genome.

[0299] The invention includes an expression construct for producing a recombinant polypeptide comprising a secretion signal peptide operably linked to a heterologous protein or polypeptide of interest. The expression construct may comprise: a nucleic acid sequence encoding a secretion signal peptide identical or substantially identical to the amino acid sequence set forth in SEQ ID NO: 11, operably linked to a nucleic acid sequence encoding the protein or polypeptide of interest. In some embodiments, the nucleic acid sequence encoding a secretion signal peptide identical or substantially identical to the amino acid sequence set forth in SEQ ID NO: 11 has a sequence that is identical or substantially identical to the nucleic acid sequence set forth as SEQ ID NO: 12. In some embodiments, the nucleic acid sequence has at least 85%, at least 90%, or at least 95% sequence identity to the nucleic acid sequence set forth as SEQ ID NO: 12.

[0300] The expression vector may comprise an expression construct comprising a nucleic acid sequence encoding a secretion signal peptide identical or substantially identical to the amino acid sequence set forth in SEQ ID NO: 11, operably linked to a nucleic acid sequence encoding the protein or polypeptide of interest. In some embodiments, the nucleic acid sequence encoding a secretion signal peptide identical or substantially identical to the amino acid sequence set forth in

SEQ ID NO: 11 has a sequence that is identical or substantially identical to the nucleic acid sequence set forth as SEQ ID NO: 12. In some embodiments, the nucleic acid sequence has at least 85%, at least 90%, or at least 95% sequence identity to the nucleic acid sequence set forth as SEQ ID NO: 12.

[0301] Methods for expressing heterologous proteins, including regulatory sequences (e.g., promoters, secretion leaders, and ribosome binding sites) useful in the methods of the invention in host strains, including *Pseudomonas* host strains, may be used as disclosed herein throughout. Such methods are known in the art and are described, e.g., in U.S. Patent No. 7,618,799, “Bacterial leader sequences for increased expression,” in U.S. Pat. No. 7,985,564, “Expression systems with Sec-system secretion,” in U.S. Pat. Nos. 9,394,571 and 9,580,719, both titled “Method for Rapidly Screening Microbial Hosts to Identify Certain Strains with Improved Yield and/or Quality in the Expression of Heterologous Proteins,” U.S. Pat. No. 9,458,487 and U.S. Pat. No. 9,453,251, both entitled “Expression of mammalian proteins in *Pseudomonas fluorescens*,” U.S. Pat. No. 8,603,824, “Process for Improved Protein Expression by Strain Engineering,” and U.S. Pat. No. 8,530,171, “High Level Expression of Recombinant Toxin Proteins,” each incorporated herein by reference in its entirety. In embodiments, a secretion leader used in the context of the present invention is a secretion leader as disclosed in any of U.S. Pat. Nos. 7, 618,799, 7,985,564, 9,394,571, 9,580,719, 9,453,251, 8,603,824, and 8,530,171. These patents also describe bacterial host strains useful in practicing the methods herein, that have been engineered to overexpress folding modulators or wherein protease mutations have been introduced, in order to increase heterologous protein expression. In embodiments, an expression host cell used in the methods of the invention is any described herein.

[0302] Exemplary Embodiments

[0303] 1. A recombinant gram-negative bacterial host cell for recombinant protein expression, wherein the host cell is:

- (a) deficient in a first protease activity, wherein the first protease activity is tail-specific protease activity, wherein the deficient first protease activity results from a mutation in at least one gene encoding a tail-specific protease;
- (b) deficient in a second protease activity, wherein the second protease activity is murein DD-endopeptidase activity, wherein the deficient second protease activity results from a mutation in at least one gene encoding a murein DD-endopeptidase.

[0304] 2. The recombinant gram-negative bacterial host cell of embodiment 1, wherein the host cell further: (c) is deficient in at least one additional protease activity, wherein the deficient additional protease activity results from a mutation in at least one gene encoding an additional protease, wherein the additional protease is different from the proteases of (a) and (b);

(d) is deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor;

(e) overexpresses one or more inactivated protease;

(f) overexpresses one or more folding modulator; or

(g) any combination of (c), (d), (e) and (f).

[0305] 3. The recombinant gram-negative bacterial host cell of embodiment 1 or 2, wherein the deficient tail-specific protease activity results from a mutation in a gene encoding one or more of: (i) a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33, a homologue of SEQ ID NO: 33, or a Prc1 tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 33; (ii) a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35, a homologue of SEQ ID NO: 35, or a Prc2 tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 35; or (iii) a Tsp tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 71, a homologue of SEQ ID NO: 71, or an Tsp tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 71.

[0306] 4. The recombinant gram-negative bacterial host cell of any one of embodiments 1-3, wherein the deficient murein DD-endopeptidase activity results from a mutation in a gene encoding one or more of:

(i) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 1, a homologue of SEQ ID NO: 1, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 1;

(ii) a MepM murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 63, a homologue of SEQ ID NO: 63, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 63;

(iii) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 65, a homologue of SEQ ID NO: 65, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 65; and

(iv) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 66, a homologue of SEQ ID NO: 66, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 66.

[0307] 5. The recombinant gram-negative bacterial host cell of any one of embodiments 2-4, wherein: the host cell of (c) is deficient in 1 to 10 different additional protease activities; the host cell of (d) is deficient in 1-5 different autolytic factor activities; the host cell of (e) overexpresses 1 to 10 different inactivated proteases, wherein each inactivated protease is

different; the host cell of (f) overexpresses 1-10 different folding modulators, or any combination thereof.

[0308] 6. The recombinant gram-negative bacterial host cell of any one of embodiments 2-5, wherein:

the one or more deficient additional protease activity of (c) results from a mutation of at least one gene encoding an additional protease independently selected from: a serralyisin precursor, a membrane-localized protease, a murein L,D transpeptidase, a hemolysin precursor, a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor, a periplasmic serine endoprotease, an AAA+ family proteolytic machine, and a murein DD-endopeptidase different from that of (a); the one or more deficient autolytic factor activity of (d) results from a mutation of at least one gene encoding an autolytic factor independently selected from: an S-type pyocin, a linear gramicidin synthase subunit D, a hemolysin precursor, a leukotoxin, and a porin; the one or more inactivated protease of (e) is a mutant periplasmic serine endoprotease; and the one or more folding modulator of (f) is a disulfide isomerase.

[0309] 7. The recombinant gram-negative bacterial host cell of embodiment 6, wherein: the serralyisin precursor is selected from:

a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9;

a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 47; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 47; and a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 47;

the membrane-localized protease is an HtpX having the amino acid sequence set forth as SEQ ID NO: 39, a homologue of the HtpX having the amino acid sequence set forth as SEQ ID NO: 39, or an HtpX related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 39;

the murein L,D transpeptidase is a murein L,D transpeptidase having the amino acid sequence set forth as SEQ ID NO: 41, a homologue of the murein L,D transpeptidase having the amino acid sequence set forth as SEQ ID NO: 41, or a murein L,D transpeptidase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 41;

the hemolysin precursor is a hemolysin precursor having the amino acid sequence set forth as SEQ ID NO: 43, a homologue of the hemolysin precursor having the amino acid sequence set

forth as SEQ ID NO: 43, or a hemolysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 43;

the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor is a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45, a homologue of the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45, or a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 45;

the periplasmic serine endoprotease is selected from:

a DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a homologue of the DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a DegP2 related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 31;

a DegP having the amino acid sequence set forth as SEQ ID NO: 69; a homologue of the DegP having the amino acid sequence set forth as SEQ ID NO: 69; a DegP related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 69;

a DegP having the amino acid sequence set forth as SEQ ID NO: 62; a homologue of the DegP having the amino acid sequence set forth as SEQ ID NO: 62; and a DegP related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 62;

the AAA+ family proteolytic machine comprises: an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38;

the murein DD-endopeptidase is selected from:

a *P. fluorescens* MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 3; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 3; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 3;

a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 64;

a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 67;

a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68; and
a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 68;

the S-type Pyocin is an S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49, a homologue of the S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49, or an S-type Pyocin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 49;

the linear gramicidin synthase is a linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51, a homologue of the linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51, or a linear gramicidin synthase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 51;

the leukotoxin is a leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53, a homologue of the leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53, or a leukotoxin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 53;

the ShlB hemolysin transporter is an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55, a homologue of the an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55, or an ShlB hemolysin transporter related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 55;

each of the one or more overexpressed inactivated proteases is independently selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2-related protein comprising an amino acid

substitution or disruption of a DegP2 having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of an amino acid at a position corresponding to any one of 131 (His), 134 (Asp) and 236 (Ser) (SEQ ID NO: 62, numbering including leader sequence 1-26), or respective positions 105, 108, and 210, when excluding the leader sequence; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr S210A; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr H105R; and an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of any one or more amino acid at a position corresponding to any one of: 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234 of SEQ ID NO: 31; and each of the one or more folding modulators is independently selected from: a disulfide bond isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 60, 76, 77, 78, 80, and 81; a homologue of a disulfide bond isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 60, 76, 77, 78, 80, and 81; a disulfide bond isomerase-related protein having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as any one of SEQ ID NOS: 60, 76, 77, 78, 80, and 81; a protein disulfide isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 27 and 82-98; a homologue of a protein disulfide isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 27 and 82-98; and a protein disulfide isomerase-related protein having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NOS: 27 and 82-98.

[0310] 8. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the mutation is in a coding sequence or noncoding sequence of the corresponding gene,

and wherein the mutation is independently selected from: (i) a complete gene deletion, (ii) a partial gene deletion, (iii) a missense mutation, (iv) a nonsense mutation, (v) a frameshift mutation, (vi) an insertion, and (vii) any combination of (ii), (iii), (iv), (v) and (vi).

[0311] 9. The recombinant gram-negative bacterial host cell of embodiment 8, wherein the missense mutation of (iii) results in a conservative or non-conservative amino acid substitution.

[0312] 10. The recombinant gram-negative bacterial host cell of embodiment 8 or 9, wherein the noncoding sequence is a regulatory sequence.

[0313] 11. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is the activity of: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 protease amino acid sequence set forth as SEQ ID NO: 5.

[0314] 12. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is: a MepS2 having the amino acid sequence set forth as SEQ ID NO: 7; a homologue of the MepS2 having the amino acid sequence set forth as SEQ ID NO: 7; or a MepS2 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS2 protease amino acid sequence set forth as SEQ ID NO: 7.

[0315] 13. The recombinant gram-negative bacterial host cell of embodiment 11 or embodiment 12, wherein the gram-negative bacterial host cell is a *Pseudomonad*.

[0316] 14. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the gram-negative bacterial host cell is a *Pseudomonad*, and the deficient first protease activity results from a mutation of a coding sequence and/or noncoding sequence of a gene encoding *P. fluorescens* Prc1 and/or a mutation of a coding sequence and/or noncoding sequence of a gene encoding *P. fluorescens* Prc2.

[0317] 15. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the second protease activity is deficient due to a mutation that results in a conservative or non-conservative substitution in an active site amino acid or an allosteric site amino acid of a protease having the second protease activity.

[0318] 16. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the deficient second protease activity results from at least one mutation of the second protease gene, wherein the mutation results in a disruption of the amino acid sequence at a position corresponding to: (i) any one or more of residues 134 to 145 of SEQ ID NO: 1; (ii) any one or more of residues 319 to 411 of SEQ ID NO: 1; (iii) one or more of residues any 361 to

378 of SEQ ID NO: 1; (iv) any one or more residue selected from 248, 319, 330, 332, 334, 337, 378, 410, and 411 of SEQ ID NO: 1; or any combination of (i), (ii), (iii), and (iv).

[0319] 17. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the bacterial host cell is *Pseudomonas fluorescens*, wherein the deficient second protease activity results from a gene mutation that results in an amino acid substitution of SEQ ID NO: 1 selected from: Y248stop, G332S, D334N, A337T, H411Y, P410L, and any conservative or non-conservative amino acid substitution of any one of R319, H330, D334, H378, and H411.

[0320] 18. The recombinant gram-negative bacterial host cell of any preceding embodiment, wherein the host cell is capable of high-density cell growth in culture.

[0321] 19. The recombinant gram-negative bacterial host cell of embodiment 18, wherein the high-density cell growth in culture comprises growth to OD₅₇₅ of about 80 to about 300.

[0322] 20. The recombinant gram-negative bacterial host cell of embodiment 18 or 19, wherein the high-density cell growth in culture is increased in comparison to a control cell by about 2-fold to about 15-fold.

[0323] 21. The recombinant gram-negative bacterial host cell of embodiment 20, wherein the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from:

- (i) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional;
- (ii) a recombinant gram-negative bacterial host cell deficient in the first protease activity, the second protease activity, and an additional protease activity as recited in 2(c), and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity of 2(c) that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and
- (iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell.

[0324] 22. The recombinant gram-negative bacterial host cell of embodiment 21, wherein the additional protease activity of 2(c) is an activity of a serralyisin precursor having the amino acid

sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9.

[0325] 23. The recombinant gram-negative bacterial host cell of any preceding embodiment, further comprising at least one expression construct, each expression construct comprising at least one nucleic acid sequence encoding a recombinant protein of interest.

[0326] 24. The recombinant gram-negative bacterial host cell of embodiment 23, wherein the recombinant protein of interest is native or heterologous to the recombinant gram-negative bacterial host cell.

[0327] 25. The recombinant gram-negative bacterial host cell of embodiment 23, wherein the recombinant protein of interest is selected from: an antibody, antibody fragment, or derivative of an antibody or antibody fragment; an antibody-based drug, a non-antibody binding protein (e.g., an antibody mimetic, including, but not limited to, an alphabody, an iBody, an affibody, an affilin, an affitin, or an anticalin), a reagent protein; a vaccine antigen; a therapeutic protein or enzyme; non-natural protein; a pathogen protein or derivative thereof; a microbial toxin, a lipoprotein; an extracellular receptor or ligand; a protease; a kinase; a blood protein; a chemokine; a cytokine; a bone morphogenic protein; an anticoagulant; a blood factor; a bone morphogenetic protein; an engineered protein scaffold; an enzyme, e.g., a biocatalytic enzyme; a growth factor; an interferon; an interleukin; a thrombolytic agent; a hormone; and a TGF-beta family member protein.

[0328] 26. The recombinant gram-negative bacterial host cell of any one of embodiments 23-25, wherein the recombinant protein of interest is human, murine, rat, rabbit, guinea pig, camelid, shark, avian, yeast, fungal, gram-negative bacterial, or gram-positive bacterial.

[0329] 27. The recombinant gram-negative bacterial host cell of embodiment 25 or 26, wherein the antibody, antibody fragment, or derivative thereof is selected from: a monoclonal antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; modified antibody, a bispecific antibody, a chimeric antibody; a diabody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a tribody; an intrabody; a nanobody; a small modular immunopharmaceutical (SMIP); an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody, an avian antibody (e.g., a chicken antibody), a VHH-containing antibody; a F(ab); a F(ab)'; F(ab)'2; scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment (e.g., generated by reducing the hinge region disulfide bonds of IgG); an Fc fusion protein (e.g.,

comprising the Fc domain of IgG fused together with a protein or peptide of interest); a domain antibody; a VL; a VNAR; a VH; and a VHH.

[0330] 28. The recombinant gram-negative bacterial host cell of embodiment 27, wherein the VHH-containing antibody is a VHH concatenated antibody.

[0331] 29. The recombinant gram-negative bacterial host cell of any one of embodiments 25 to 28, wherein the antibody, antibody fragment, or derivative thereof, binds to a target selected from: a cytokine; a chemokine; a drug; a cell-surface protein, e.g., a receptor, cell-surface marker, pathogen surface-protein, etc.; a growth factor; a growth factor receptor; immune checkpoint molecule, and a blood factor.

[0332] 30. The recombinant gram-negative bacterial host cell of any one of embodiments 25 to 29, wherein the antibody, antibody fragment, or derivative thereof is a Fab'.

[0333] 31. The recombinant gram-negative bacterial host cell of embodiment 30, wherein the Fab' binds to a target selected from: Carcinoembryonic antigen (CEA); CD22; fibrin II, beta chain; TNF-alpha; and NCA-90 (granulocyte antigen).

[0334] 32. The recombinant gram-negative bacterial host cell of any one of embodiments 25 to 31, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least one nucleic acid sequence encoding a heavy chain, at least one nucleic acid sequence encoding a light chain, or both, wherein the heavy chain is full-length or a heavy chain fragment, and the light chain is full-length or a light chain fragment.

[0335] 33. The recombinant gram-negative bacterial host cell of embodiment 32, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least two nucleic acid sequences, each encoding a heavy chain.

[0336] 34. The recombinant gram-negative bacterial host cell of embodiment 32 or 33, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from the same mRNA transcript.

[0337] 35. The recombinant gram-negative bacterial host cell of embodiment 32 or 33, wherein the at least one expression construct encoding the antibody, an antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from different mRNA transcripts.

[0338] 36. The recombinant gram-negative bacterial host cell of embodiment 34 or 35, wherein each heavy chain-encoding nucleic acid sequence and each light chain-encoding nucleic

acid sequence is individually operably linked to an independently selected nucleic acid sequence encoding a periplasmic secretion signal.

[0339] 37. The recombinant gram-negative bacterial host cell of embodiment 36, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NO: 11, 13, 15, or 17.

[0340] 38. The recombinant gram-negative bacterial host cell of any one of embodiments 34 to 37, wherein the expression construct comprises: a nucleic acid sequence encoding an antibody heavy chain, operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NOS: 11, 13, 15, or 17; a nucleic acid sequence encoding a light chain, operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NOS: 11, 13, 15, or 17; or both.

[0341] 39. The recombinant gram-negative bacterial host cell of any one of embodiments 25 to 38, wherein the antibody, antibody fragment, or derivative thereof is humanized.

[0342] 40. The recombinant gram-negative bacterial host cell of any one of embodiments 30 to 39, wherein the Fab' is certolizumab.

[0343] 41. The recombinant gram-negative bacterial host cell of embodiment 40, wherein the Fab' heavy chain has the amino acid sequence set forth as SEQ ID NO: 21, and the Fab' light chain has the amino acid sequence set forth as SEQ ID NO: 23.

[0344] 42. The recombinant gram-negative bacterial host cell of any one of embodiments 32-41, wherein the nucleic acid sequence encoding the heavy chain is operably linked to a nucleic acid sequence encoding a secretion leader having the amino acid sequence set forth as SEQ ID NO: 11, and the nucleic acid sequence encoding the light chain is operably linked to a nucleic acid sequence encoding a secretion leader having the amino acid sequence set forth as SEQ ID NO: 13.

[0345] 43. The recombinant gram-negative bacterial host cell of any one of embodiments 1-42, wherein the host cell is deficient in:

- (i) the first protease activity;
- (ii) the second protease activity;
- (iii) the activity of a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9, a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9, or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9;
- (iv) an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU

related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and

(v) an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38.

[0346] 44. The recombinant gram-negative bacterial host cell of embodiment 43, wherein the host cell further overexpresses an exogenous inactivated DegP, wherein the inactivated DegP is selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 derived from the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP2 derived from a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from a DegP2 having at least 60% similarity or at least 60% identity to amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from a DegP2 having at least 60% similarity or at least 60% identity to amino acid sequence set forth as SEQ ID NO: 62; and each of the proteases having the amino acid sequence set forth as SEQ ID NO: 31 comprising a conservative or nonconservative amino acid substitution or disruption of any one or more of positions: 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234.

[0347] 45. The recombinant gram-negative bacterial host cell of embodiment 43 or 44, wherein the host cell overexpresses an exogenous disulfide isomerase selected from any one of: a disulfide isomerase having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NO: 27, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73, and a homologue of a disulfide isomerase having the amino acid sequence set forth as SEQ ID NO: 27, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, and 73.

[0348] 46. The recombinant gram-negative bacterial host cell of any one of embodiments 1-45, wherein the recombinant gram-negative bacterial host cell is selected from: a *Pseudomonad* host cell; an *E. coli* host cell; and a *Vibrio* host cell.

[0349] 47. The recombinant gram-negative bacterial host cell of embodiment 46, wherein the *Pseudomonad* host cell is a *Pseudomonas* host cell.

[0350] 48. The recombinant gram-negative bacterial host cell of embodiment 47, wherein the *Pseudomonas* host cell is *P. fluorescens*, *P. putida*, or *P. aeruginosa*.

[0351] 49. The recombinant gram-negative bacterial host cell of embodiment 47 or 48, wherein the cell is:

- (i) lsc::lacIQ1;
- (ii) Prc1-
- (ii) Prc2-
- (iii) HslU-
- (iv) HslV-
- (v) MepM1-
- (vi) PyrF-

and (vii) deficient in a serralysin precursor that is: a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; wherein the serralysin precursor deficiency results from a mutation in a gene encoding the serralysin precursor.

[0352] 50. The recombinant gram-negative bacterial host cell of embodiment 49, wherein the cell is *P. fluorescens*, the Prc1 has the amino acid sequence set forth in SEQ ID NO: 33, the Prc2 has the amino acid sequence set forth in (SEQ ID NO: 35), the HslU has the amino acid sequence set forth in (SEQ ID NO: 37), the HslV has the amino acid sequence set forth in (SEQ ID NO: 38), the MepM1 has the amino acid sequence set forth in (SEQ ID NO: 1), and the serralysin precursor has the amino acid sequence set forth as SEQ ID NO: 9.

[0353] 51. The recombinant gram-negative bacterial host cell of embodiment 50, further comprising an expression vector comprising a nucleic acid sequence encoding DegP2 S219A (SEQ ID NO: 29).

[0354] 52. The recombinant gram-negative bacterial host cell of embodiment 50 or 51, further comprising an expression vector comprising a nucleic acid sequence encoding disulfide isomerase PDIA6 (SEQ ID NO: 27).

[0355] 53. The recombinant gram-negative bacterial host cell of any one of embodiments 1-52, further comprising an expression vector encoding a recombinant protein.

[0356] 54. The recombinant gram-negative bacterial host cell of embodiment 53, wherein the expression vector encodes a Fab'.

[0357] 55. The recombinant gram-negative bacterial host cell of embodiment 54, wherein the expression vector comprising the nucleic acid sequence encoding DegP2 S219A or disulfide isomerase PDIA6 further comprises a nucleic acid sequence encoding the Fab'.

[0358] 56. The recombinant gram-negative bacterial host cell of embodiment 54 or 55, wherein the Fab' heavy chain is encoded by SEQ ID NO: 21, and the Fab' light chain is encoded by SEQ ID NO: 23.

- [0359] 57. The recombinant gram-negative bacterial host cell of embodiment 1, wherein the recombinant gram-negative bacterial host cell is a *Pseudomonad* having the genotype of strain STR94975, STR94976, or STR94977.
- [0360] 58. The recombinant gram-negative bacterial host cell of embodiment 57, further comprising the expression construct or constructs of STR94975, STR94976, or STR94977, for use in producing a recombinant anti-TNF-alpha Fab'.
- [0361] 59. A method for producing a recombinant protein of interest comprising: (a) recovering the recombinant protein of interest from a recombinant gram-negative bacterial host cell of any one of embodiments 1-57 cultured under suitable fermentation conditions, wherein the recombinant gram-negative host cell is transformed with a plasmid comprising a nucleic acid encoding the recombinant protein of interest.
- [0362] 60. The method of embodiment 59, wherein transcription of the nucleic acid sequence encoding the recombinant protein of interest is regulated by an inducible promoter.
- [0363] 61. The method of embodiment 60, wherein the inducible promoter is selected from: a tac promoter, a mannitol promoter, a Pben, a T7 promoter, a lac promoter, a T5 promoter, a xylose promoter, and an arabinose promoter.
- [0364] 62. The method of any one of embodiments 59-61, wherein the recombinant gram-negative bacterial host cell can grow to high cell density.
- [0365] 63. The method of embodiment 62, wherein the high cell density comprises an OD₅₇₅ of about 80 to about 300.
- [0366] 64. The method of any one of embodiments 58-63, wherein the suitable fermentation conditions comprise induction of the inducible promoter at: an OD₅₇₅ of about 80 to about 160, a culture pH of about 5.8 to about 7.0, a temperature of about 28-33 deg C, fed-batch, and a titer range of about 0.2 to about 5 g/L.
- [0367] 65. The method of embodiment 64, wherein the inducible promoter is induced by IPTG, and wherein the IPTG is added to a final concentration of about 0.08 – 0.3mM.
- [0368] 66. The method of embodiment 65, wherein the IPTG is added to a final concentration of about 0.2 mM.
- [0369] 67. The method of any one of embodiments 63-66, wherein induction is carried out at a culture pH of about 6.0 to about 6.5.
- [0370] 68. The method of any one of embodiments 63-67, wherein induction is carried out at a temperature of about 28-33 deg C.
- [0371] 69. The method of embodiment 68, wherein induction is carried out at a temperature of about 32 deg C.

- [0372] 70. The method of any one of embodiments 59-69, wherein the recombinant gram-negative bacterial host cell grows to a cell density that is increased in comparison to a control cell grown under the same fermentation conditions.
- [0373] 71. The method of embodiment 70, wherein the increase in cell density is about 2-fold to about 15-fold.
- [0374] 72. The method of any one of embodiments 59-71, further comprising: (b) measuring the yield of intact, soluble, and/or active, recombinant protein of interest recovered from the recombinant gram-negative bacterial host cell.
- [0375] 73. The method of embodiment 72, wherein the measured yield of intact, soluble, and/or active, recombinant protein is about 0.1 to about 10 g/L.
- [0376] 74. The method of embodiment 72 or 73, further comprising: (c) measuring the yield of recombinant protein of interest recovered from a control cell that is intact, soluble, active, or a combination thereof.
- [0377] 75. The method of embodiment 74, further comprising (d) comparing the yield measured in step (b) to the yield measured in step (c).
- [0378] 76. The method of embodiment 75, wherein the yield measured in step (b) is about 2-fold to about 100-fold higher than that measured in step (c).
- [0379] 77. The method of any one of embodiments 70-76, wherein the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from:
- [0380] (i) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional;
- [0381] (ii) a recombinant gram-negative bacterial host cell deficient in the first protease activity, the second protease activity, and an additional protease activity as recited in 2(a), and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity of 2(a) that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and
- [0382] (iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell.

- [0383] 78. The recombinant gram-negative bacterial host cell of any one of embodiments 1-45, or any one of embodiments 47-77, wherein the gram-negative bacterial host cell is not *E. coli*.
- [0384] 79. recombinant polypeptide comprising: a secretion signal peptide operably linked to a heterologous protein or polypeptide of interest, wherein the secretion signal peptide has the amino acid sequence set forth in SEQ ID NO: 11.
- [0385] 80. The polypeptide of embodiment 79, wherein the protein or polypeptide of interest is selected from: an antibody, antibody fragment, or a derivative of an antibody or an antibody fragment; an enzyme; a cytokine; a chemokine; a growth factor; a fusion protein; and a vaccine antigen.
- [0386] 81. The polypeptide of embodiment 79 or 80, wherein the antibody, antibody fragment, or a derivative of an antibody or antibody fragment is selected from: a monoclonal antibody; a full chain antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; a modified antibody; a variable-region only antibody fragment; a bispecific antibody, a chimeric antibody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a dibody; an intrabody; a nanobody; a small modular immunopharmaceutical; an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody (VNAR); an avian antibody; a VHH; a VHH-containing antibody; a VHH concatemer; a F(ab); a F(ab)'; F(ab)'2; an scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment; an Fc fusion protein; a domain antibody; a VL; and a VH.
- [0387] 82. The polypeptide of embodiment 80 or 81, wherein the antibody, antibody fragment, or derivative of the antibody or antibody fragment is humanized.
- [0388] 83. The polypeptide of embodiment 80, wherein the enzyme is a therapeutic enzyme.
- [0389] 84. The polypeptide of embodiment 83, wherein the therapeutic enzyme is selected from: a peptidase; a lactase; an amylase; a PEP; a digestive enzyme; a uricase; a rhodanase; a urokinase; a streptokinase; a staphylokinase; a phenylase; a sacrosidase; a lysozyme; a chitinase; a ribonuclease; a glutaminase; an arginase; a vibrilase; a chondroitinase; a hyaluronidase; a galactosidase; a glucuronidase; a glucocerebrosidase; a thymidine phosphorylase; a carbonic anhydrase; a uricase thiosulfate-cyanide; a sulfurtransferase; a phosphothioesterase; an alcohol oxidase; an alcohol dehydrogenase; an asparaginase; a glutamine synthase; an adenosine deaminase; bovine pegademase; alglucerase; dornase alpha; imiglucerase; sacrosidase; rasburicase; agalsidase beta; and nattokinase.
- [0390] 85. The polypeptide of embodiment 80, wherein the fusion protein is selected from: an enzyme fusion protein; a protein A fusion protein; an albumin fusion protein; a thioredoxin fusion protein; a ubiquitin fusion protein; a streptavidin fusion protein; a maltose binding protein

fusion protein; a chitin binding protein fusion protein; a SUMO fusion protein; and a glutathione-S-transferase fusion protein.

[0391] 86. The polypeptide of any one of embodiments 79-85, further comprising a linker.

[0392] 87. The polypeptide of any one of embodiments 79-86, further comprising a cleavage domain.

[0393] 88. The polypeptide of any one of embodiments 79-87, wherein the secretion signal peptide directs expression of the protein or polypeptide of interest to the periplasm or the extracellular space of a prokaryotic host cell.

[0394] 89. The polypeptide of embodiment 88, wherein the prokaryotic host cell is a gram-negative bacterium.

[0395] 90. The polypeptide of embodiment 88, wherein the prokaryotic host cell is a gram-positive bacterium.

[0396] 91. The polypeptide of embodiment 89, wherein the gram-negative bacterium is a *Pseudomonad*, *V. natriegens*, or *E. coli*.

[0397] 92. The polypeptide of embodiment 90, wherein the gram-positive bacterium is a *Corynebacterium* or a *Bacillus*.

[0398] 93. A method of producing a protein or polypeptide of interest in a prokaryotic host cell, the method comprising: producing the protein or polypeptide of interest in the periplasm of a prokaryotic host cell cultured in a cell culture growth medium, wherein the prokaryotic host cell comprises an expression construct comprising a nucleic acid encoding a recombinant polypeptide comprising the protein or polypeptide of interest operably linked to a secretion signal peptide that directs expression of the protein or polypeptide of interest to the periplasm of the prokaryotic host cell, wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 11, and wherein the secretion signal peptide is not native to the protein or polypeptide of interest.

[0399] 94. The method of embodiment 93, further comprising isolating the produced protein or polypeptide of interest.

[0400] 95. The method of embodiment 94, wherein the protein or polypeptide of interest is selected from: an antibody, antibody fragment, or a derivative of an antibody or an antibody fragment; an enzyme; a cytokine; a chemokine; a growth factor; a fusion protein; and a vaccine antigen.

[0401] 96. The method of embodiment 95, wherein the antibody, antibody fragment, or a derivative of an antibody or an antibody fragment is selected from: a monoclonal antibody; a full chain antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; a modified antibody; a variable-region only antibody fragment; a bispecific antibody, a chimeric antibody; a triabody; a tetrabody; a

minibody; a linear antibody; a chelating recombinant antibody; a bibody; a dibody; an intrabody; a nanobody; a small modular immunopharmaceutical; an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody (VNAR); an avian antibody; a VHH; a VHH-containing antibody; a VHH concatemer; a F(ab); a F(ab)'; F(ab)'2; an scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment; an Fc fusion protein; a domain antibody; a VL; and a VH.

[0402] 97. The method of embodiment 95 or 96, wherein the antibody, antibody fragment, or derivative of the antibody or antibody fragment is humanized.

[0403] 98. The method of embodiment 95, wherein the enzyme is a therapeutic enzyme.

[0404] 99. The method of embodiment 98, wherein the therapeutic enzyme is selected from: a peptidase; a lactase; an amylase; a PEP; a digestive enzyme; a uricase; a rhodanase; a urokinase; a streptokinase; a staphylokinase; a phenylase; a sacrosidase; a lysozyme; a chitinase; a ribonuclease; a glutaminase; an arginase; a vibrilase; a chondroitinase; a hyaluronidase; a galactosidase; a glucuronidase; a glucocerebrosidase; a thymidine phosphorylase; a carbonic anhydrase; a uricase thiosulfate-cyanide; a sulfurtransferase; a phosphothioesterase; an alcohol oxidase; an alcohol dehydrogenase; an asparaginase; a glutamine synthase; an adenosine deaminase; bovine pegademase; alglucerase; dornase alpha; imiglucerase; sacrosidase; rasburicase; agalsidase beta; and nattokinase.

[0405] 100. The method of embodiment 95, wherein the fusion protein is selected from: an enzyme fusion protein; a protein A fusion protein; an albumin fusion protein; a thioredoxin fusion protein; a ubiquitin fusion protein; a streptavidin fusion protein; a maltose binding protein fusion protein; a chitin binding protein fusion protein; a SUMO fusion protein; and a glutathione-S-transferase fusion protein.

[0406] 101. The method of any one of embodiments 93-100, wherein the nucleic acid encodes a linker.

[0407] 102. The method of embodiment 101, wherein the linker comprises a cleavage domain.

[0408] 103. The method of any one of embodiments 93-102, wherein the prokaryotic host cell is a gram-negative bacterium.

[0409] 104. The method of any one of embodiments 93-102, wherein the prokaryotic host cell is a gram-positive bacterium.

[0410] 105. The method of embodiment 93, wherein the gram-negative bacterium is a *Pseudomonad*, *V. natriegens*, or *E. coli*.

[0411] 106. The method of embodiment 94, wherein the gram-positive bacterium is a *Corynebacterium* or a *Bacillus*.

[0412] 107. An expression vector comprising a nucleic acid sequence encoding a recombinant polypeptide of any one of embodiments 79-92.

[0413] 108. A prokaryotic host cell comprising an expression vector of embodiment 107.

[0414] 109. The prokaryotic host cell of embodiment 108, wherein the prokaryotic host cell is a gram-negative bacterium.

[0415] 110. The prokaryotic host cell of embodiment 108, wherein the prokaryotic host cell is a gram-positive bacterium.

[0416] 111. The prokaryotic host cell of embodiment 109, wherein the gram-negative bacterium is a *Pseudomonad*, *V. natriegens*, or *E. coli*.

[0417] 112. The prokaryotic host cell of embodiment 110, wherein the gram-positive bacterium is a *Corynebacterium* or a *Bacillus*.

[0418] 113. The prokaryotic host cell of any one of embodiments 108-112, wherein the nucleic acid sequence encoding the recombinant polypeptide is optimized for expression in the prokaryotic host cell.

[0419] 114. Use of a recombinant polypeptide of any one of embodiments 79-92, an expression vector of embodiment 107, or a prokaryotic host cell of any one of embodiments 108-113, for expressing a protein or polypeptide of interest in the periplasm or the extracellular space of a prokaryotic host cell.

[0420] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

EXAMPLES

[0421] The following examples are given for the purpose of illustrating various embodiments of the disclosure and are not meant to limit the present disclosure in any fashion. The present examples, along with the methods described herein are presently representative embodiments, are exemplary, and are not intended as limitations on the scope. Changes therein and other uses which are encompassed within the spirit of the disclosure as defined by the scope of the claims will occur to those skilled in the art.

[0422] Example 1. Recombinant Protein Expression in Protease-Deficient Bacterial Host Cells

[0423] Screening of protease-deficient host cell strains for production of intact recombinant protein was carried out at 0.5 mL scale. In each of fifteen different protease-deficient *P. fluorescens* strains, each of ten anti-TNF Fab' (certolizumab) overexpression plasmids were tested. Each of the ten overexpression plasmids (constructs 1-10) contained an expression construct encoding the anti-TNF-alpha Fab' heavy chain (HC, SEQ ID NO: 21, encoded by SEQ ID NO: 22), and the anti-TNF-alpha Fab' light chain (LC, SEQ ID NO: 23, encoded by SEQ ID NO: 24), under control of the same promoter, Ptac. Constructs 1-9 each contained a plasmid comprising an expression construct having sequences encoding, from 5'-3': Leader 1 (L1) – HC (SEQ ID NO: 21, encoded by SEQ ID NO: 22) – Leader 2 (L2) – LC (SEQ ID NO: 23, encoded by SEQ ID NO: 24) – DsbC (SEQ ID NO: 60, encoded by SEQ ID NO: 79) (a co-transcriptional *P. fluorescens* DsbC protein disulfide isomerase). Construct 10 contained p688-005, which did not co-express DsbC. Like constructs 1-9, construct 10 comprised sequences encoding, from 5'-3', Leader 1 (L1) – HC (HC, SEQ ID NO: 21, encoded by SEQ ID NO: 22) – Leader 2 (L2) – LC (SEQ ID NO: 23, encoded by SEQ ID NO: 24). The ten plasmids were the same except for the differences in secretion signal sequences and differences noted for construct 10. In each of constructs 1-9, L1 was a different secretion signal; constructs 3 and 10 had the same secretion signal. In each of constructs 1-10, L2 was the Azu periplasmic secretion signal (SEQ ID NO: 13, encoded by SEQ ID NO: 14). In construct 8, L1 was the Slmt secretion signal (SEQ ID NO: 11, encoded by SEQ ID NO: 12). Each light and heavy chain gene comprised a high translation efficiency ribosome binding sequence (having 100% of the activity of the canonical Shine-Dalgarno sequence, SEQ ID NO: 59) upstream of the start codon.

[0424] The expression plasmids were transformed into the *P. fluorescens* host strains in an array format. The transformation reaction was initiated by mixing *P. fluorescens* competent cells and plasmid DNA. A 25 µL aliquot of the mixture was transferred to a 96-multi-well Nucleovette® plate (Lonza). Electroporation was carried out using the Nucleofector™ 96-well Shuttle™ system (Lonza AG), and the electroporated cells were subsequently transferred to a fresh 96-well deep well plate, containing 500 µL M9 salts supplemented with 1% glucose medium, and trace elements. The plates were incubated at 30 °C with shaking for 48 hours, to generate seed cultures.

[0425] Ten µL aliquots of the seed cultures were transferred in duplicate into 96-well deep well plates. Each well contained 500 µL of HTP-YE medium (Teknova), supplemented with trace elements and 5% glycerol. The seed cultures, plated in the glycerol supplemented HTP media,

were incubated for 24 hours, in a shaker, at 30°C. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to each well at a final concentration of 0.3 mM to induce expression of the Fab'. After 24 hours of induction, cell density was calculated by measuring the optical density at 600 nm (OD₆₀₀). The cells were subsequently harvested, diluted 1:3 with 1X Phosphate Buffered Saline (PBS) to a final volume of 400 μ L, and frozen for later processing.

[0426] Soluble Lysate Sample Preparation for Analytical Characterization: The harvested cell samples were diluted and lysed by sonication with a Cell Lysis Automated Sonication System (CLASS, Scinomix) using a 24 probe tip horn. The lysates were centrifuged at 5,500 x g for 15 minutes at 8 °C. The supernatant was collected and labeled as the soluble fraction. The pellets were collected, resuspended in 400 μ L of 1X PBS pH 7.4 by another round of sonication, and labeled as the insoluble fraction.

[0427] Nonreducing SDS-CGE Analysis: The soluble and insoluble fractions were analyzed by HTP microchip SDS capillary gel electrophoresis using a LabChip GXII instrument (Caliper LifeSciences) with a HT Protein Express v2 chip and corresponding reagents (part numbers 760499 and 760328, respectively, Caliper LifeSciences). Samples were prepared following the manufacturer's protocol (Protein User Guide Document No. 450589, Rev. 3). Briefly, 4 μ L aliquots of either the soluble or the insoluble fraction samples was mixed with 14 μ L of buffer in 96-well polypropylene conical well PCR plates heated at 95 °C for 5 minutes, and diluted with 70 μ L deionized water. Lysates from null host strains, which were not transformed with the Fab' expression plasmid, as well as host strains transformed with construct 10, were run as control in parallel with test samples, and quantified using the system internal standard.

[0428] Fig. 1 shows the nonreducing SDS-CGE analysis of Fab' protein produced by DC1032 and four other protease-deficient *P. fluorescens* strains tested. The twelve lanes for each strain show, from left to right: far left lane - MW ladder with MW's 16, 20, 29, 48, and 68 kD; lanes numbered 1-10 – protein expressed using constructs 1-10, each encoding the Fab' heavy and light chain, and each having a nucleic acid sequence encoding a different periplasmic secretion signal operably linked to the heavy chain gene and each having a nucleic acid sequence encoding the Azu secretion signal operably linked to the light chain gene. Constructs 1-9 each co-express *P. fluorescens* derived DsbC while construct 10 does not. Lane 12 (far right lane in each set) – null host strain (with a null expression plasmid). From left to right, the first 12 lanes show Fab' expression in host strain DC1084; the second 12 lanes show Fab' protein expression in DC977; the third 12 lanes show Fab' protein expression in DC441; the fourth 12 lanes show Fab' protein expression in DC1032; and the fifth 12 lanes show Fab' protein expression in DC509. The arrow below the DC1032 lanes indicates the area of migration for the Fab' degradation products.

HPLC and LC-MS analyses of Protein L enriched Fab' confirmed that the observed fragments were derived from the Fab'.

[0429] Conclusion: In contrast with the other fourteen host strains tested, DC1032, which has Prc1, Prc2 and HslUV protease deficiencies (by gene knockout), produced a high yield of assembled Fab' (up to 130 mg/L), with reduced degradation (see arrow at right indicating Fab'). Construct 8 comprised a nucleic acid sequence encoding secretion signal Slmt (SEQ ID NO: 11) operably linked to the heavy chain gene, and a nucleic acid sequence encoding secretion signal Azu (SEQ ID NO: 13) operably linked to the light chain gene, produced the highest assembled Fab' yields, and reduced degradation.

[0430] Example 2. Restoration of Protease-Deficient Bacterial Host Cell Growth

[0431] Growth of identified host strains at large scale

[0432] *P. fluorescens* host strains identified as described in Example 1 at the 0.5 mL scale were grown at 2-liter (2L) scale (conventional bioreactor, CBR). Complete inactivation of both tail-specific protein genes (Prc) in *P. fluorescens* prevented high cell density growth in bioreactors. A similar effect previously was reported regarding *E. coli* tail-specific protease mutants, e.g., by U.S. Pat. No. 9,493,559, EP1341899 B1, "Bacterial host strains," and Hara, H. et al., 1991, each incorporated herein by reference in its entirety.

[0433] Fig. 2 shows growth curves (OD575 vs fermentation time in hours) for DC1032 host cell strains at 2L scale. Cells were grown at 32 degrees C, pH 6.5 induced with IPTG at 25.5 hours and the induction phase proceeded for 36 hours. Strain STR36306 (DC1032 + p688-048 (Fab' expression plasmid); grew to a maximum OD575 of about 27, and STR94998 (DC1032 + null plasmid, grew to a maximum OD575 of about 50). These Prc-deficient strains failed to grow beyond an OD575 of 20-50, and showed evidence of cell lysis. In contrast, strains expressing both functional copies of Prc (Prc1 and Prc2) grew to an OD575 of up to 130 in 2L bioreactors within 18-26 hours (data not shown).

[0434] E. coli strategy fails to restore high-density cell growth to Pseudomonas host strains

[0435] Previous studies showed that in *E. coli*, deletion of the gene encoding tail-specific protease Tsp/Prc (an orthologue of *Pseudomonas* Prc) inhibits growth at high density fermentation, and that inactivation of Spr (the *E. coli* analogue of *Pseudomonas* MepS1) by amino acid substitution restored *E. coli* growth to an OD of greater than or equal to 200 at 575nm (e.g., U.S. Pat. No. 9,493,559, EP1341899 B1, and Hara, H. et al., 1991). This strategy proved unsuccessful in *P. fluorescens* Prc null strains. As shown in Fig. 2, growth of Prc null strains was not restored by deficiency in MepS1, MepS2, or both. As shown, the strain STR94994, DC1032 additionally having a MepS1 deletion, grew to a maximum OD575 of about 23, the strain STR94995, DC1032 having a MepS2 deletion, grew to a maximum OD575 of about 33,

and the strain STR94996, DC1032 having deletions of both MepS1 and MepS2, grew to a maximum OD₅₇₅ of about 23. (Strains STR94994, STR94995 and STR94996 each contained p688-048).

[0436] Adaptive Laboratory Evolution of *Prc1*- and *Prc2*-deficient host cells

[0437] *Prc* deficient host strains with restored high-cell density growth were developed.

Table 4: *P. fluorescens* host strains used in Adaptive Laboratory Evolution Studies

Strain	Gene Deletions	Other
DC1032	<i>prc1</i> , <i>prc2</i> , <i>hslUV</i> , <i>pyrF</i>	<i>lsc::lacIQ1</i>
DC954	<i>prc1</i> , <i>prc2</i> , <i>pyrF</i>	<i>lsc::lacIQ1</i>
DC454	<i>pyrF</i>	<i>lsc::lacIQ1</i>

[0438] DC954 was subjected to adaptive laboratory evolution (ALE) under growth conditions that are lethal to *Prc* null strains. ALE and similar strategies have been described in the literature, e.g., in *E. coli*, by Hara, H. et al., 1991. In ALE, bacterial strains with reduced tolerance to stress (including protease-deficient mutants) are subjected to sub-optimal growth conditions - here, low salt and high temperature – that prohibit growth unless further chromosomal mutation arises.

[0439] DC954 was grown at high temperature (36 deg C, as opposed to the cells' optimal growth temperature of 30-32 deg C) on a modified hypotonic Luria-Bertani (LB) medium agar recipe similar to that described by Hara et al., 1991. **Fig. 3A** shows growth of DC954 colonies, on 1X LB agar medium in the absence of NaCl, and in the presence of uracil added to allow growth given the *pyrF* deletion, at 36 deg C. As shown, under these conditions DC954 formed colonies with background growth. In **Fig. 3B**, the left plate shows growth of DC454 (essentially wild-type) on only one-half-X LB agar medium in the absence of NaCl, with uracil, at 36 deg C. Growth was slower than on 1X LB, but these growth conditions were not lethal to DC454. The right plate in **Fig. 3B** shows growth of DC954 on one-half-X LB agar medium in the absence of NaCl, with uracil, at 36 deg C. Under these growth conditions, background growth was greatly reduced or eliminated, but several robust single colonies survived. The single colonies, as indicated, suggested cells that had adapted (evolved) to the sub-optimal growth conditions by further mutation. These colonies were picked for further characterization.

[0440] The chromosomal MepS, MepM and MepH orthologues in the evolved cells were sequenced. MepM1 was found to have acquired mutations consistent with its inactivation. The identified mutations were: Y248stop, D334N, G332S, A337T, H411Y, and P410L. These mutations restored growth at 2L scale when co-expressed with an empty vector rescuing auxotrophy to uracil. Therefore, inactivation of MepM1 restored high cell density growth to DC954.

Table 5: Growth of Evolved vs Unevolved *P. fluorescens* host strains (Fig. 4)

Strain #	Evolved	Prc	Host Cell Genotype	Plasmid	Density (OD575)
1 PF1550	Yes	Null	Evolved from DC954: Δ prc1, Δ prc2, MepM1(P410L) , Δ pyrF, lsc::lacIQ1	p688-048 (Fab' DsbC)	High (at least 140)
2 STR36306	No	Null	DC1032: Δ prc1, Δ prc2, Δ hslUV, Δ pyrF, lsc::lacIQ1	p688-048 (Fab' DsbC)	Low (less than 50)
3 PF1557	Yes	Null	Δ prc1, Δ prc2, MepM1(P410L) , Δ pyrF, lsc::lacIQ1	pDOW1169 (empty expression vector)	High (at least 150)
4 DC432	No	+	DC454: Δ pyrF	pDOW1169 (empty expression vector)	High (at least 175)

*Bolding in column 4 indicates mutation acquired by evolution.

[0441] Fig. 4 shows that evolved Prc null strain #'s 1 and 3 grew to high cell densities (OD575 of 132-150), as did unevolved Prc+ strain #4. However, unevolved Prc null strain 2 grew to an OD575 of less than 50. (See **Table 5**.) The Production phase was conducted at 32 deg C, pH 6.5 for a duration of 48 hrs.

[0442] Generation of combinatorial MepS and MepM deficiencies in a Prc null background

[0443] A series of MepS and MepM knockout mutants were generated for systematic testing in Prc-deficient *P. fluorescens* (Δ prc1 Δ prc2, Δ pyrF). Homologous regions to MepS1, MepS2, MepM1 and MepM2 were synthesized and subcloned by blunt end restriction digest into a non-*pseudomonas* replicating vector, and selected by rescued prototrophy for uracil.

[0444] Transformation was carried out electroporating 1ug of plasmid DNA into electro competent prc knockout *P. fluorescens* cell lines. Selection for integration was done by plating transformed cells onto M9 minimal media + 1mM MgCl₂ + 1.5% agar and allowed to select for 2-3 days at 30 deg C. A second recombination step was selected for by picking single colonies into LB media + 250 ug/ml uracil overnight. The following day several dilutions of the saturated culture was spread onto a 0.5X LB, 500 ug/ml 5-FOA + 250 ug/ml uracil 1.5% agar plate. This second recombination event knocks out the open reading frame, and a non-replicating plasmid is dropped from cells through 5-FOA counter-selection of the pyrF gene. Single colonies were picked into liquid 0.5X LB, 500ug/ml 5-FOA + 250ug/ml uracil and grown overnight at 30 deg C. For colony PCR, primers were designed outside of the homology arm regions above and knockouts were confirmed by size analysis on an ethidium bromide stained agarose after amplification by PCR.

[0445] Screening of combinatorial mutants

[0446] For growth phenotype analysis, the knockout strains were grown overnight in M9 media + 1mM MgCl₂ + 250ug/ml uracil (M9/Ura) to allow growth given the pyrF deletion. The following day, saturated liquid cultures were diluted 1:100 in M9/Ura followed by five 10-fold serial dilutions in M9/Ura. The cultures were then incubated under suboptimal conditions by spotting (5 ul) onto a 0.5X LB (no NaCl, 250ug/ml uracil) 1.5% agar plate and incubating at 36 deg C for 48 hours.

[0447] **Table 6** shows the combinatorial Mep deficient mutants screened. Unlike rescue of Tsp mutants by Spr mutation reported in *E. coli*, MepS1 deficiency in *P. fluorescens* unexpectedly did not restore high density growth to the Prc deficient mutant. **Fig. 5** shows the screening results. The sequentially lower dilutions were applied from the top to bottom of each row on the plate as oriented in the figure. Column 1 contains Prc-deficient mutant DC1032, which fails to grow under the sub-optimal conditions, and Row 10 shows growth of a Prc+ ΔpyrF strain (wild-type control) under the same conditions. As observed in the other rows:

[0448] Column 2 - the presence of a MepM1 deficiency successfully restores growth to the Prc deficient mutant at all titers.

[0449] Column 3 - the presence of a MepS1 deficiency eliminates the ability of a MepM1 deficiency to restore growth.

[0450] Column 4 - the presence of a MepS2 deficiency does not affect the ability of a MepM1 deficiency to restore growth.

[0451] Column 5 - the presence of MepS1 and MepS2 deficiencies eliminate the ability of a MepM1 deficiency to restore growth.

[0452] Column 6 - the presence of a MepM2 deficiency fails to restore growth to the Prc deficient mutant.

[0453] Column 7 - the presence of MepM2 and MepS1 deficiencies do not restore growth to the Prc deficient mutant.

[0454] Column 8 - the presence of MepM2 and MepS2 deficiencies do not restore growth to the Prc deficient mutant.

[0455] Column 9 - the presence of MepM2, MepS1, and MepS2 deficiencies do not restore growth to the Prc deficient mutant.

[0456] A host cell having all four deficiencies (MepM1, MepM2, MepS1, and MepS2) was not successfully isolated, suggesting lethality in the Prc deficient mutant.

[0457] The growth observed in **Fig. 5** is noted in the fourth column of **Table 6**.

Table 6: Combinatorial Mep knockout (KO) mutants screened (all Prc null)

Mep KO	ID	Genotype	Fig. 5 Result
MepS1	PF1561	Δprc1Δprc2, ΔhslUV, ΔmepS1, ΔpyrF, lsc::lacIQ1	No growth

MepS2	PF1582	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Not tested
MepS1/MepS2	PF1562	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS1 , ΔmepS2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Not tested
MepM1	PF1559	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepM1 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 2: growth ++
MepM2	PF1572	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepM2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 6: no growth
MepM1/MepM2	PF1584	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepM1 , ΔmepM2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Not tested
MepS1/MepM1	PF1588	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS1 , ΔmepM1 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 3: no growth
MepS1/MepS2/MepM1	PF1590	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS1 , ΔmepS2 , ΔmepM1 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 5: no growth
MepS1/MepS2/MepM1/MepM2	unable to isolate	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS1 , ΔmepS2 , ΔmepM1 , ΔmepM2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Not tested
MepS1/MepM2	PF1577	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS1 , ΔmepM2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 7: no growth
MepS2/MepM1	PF1560	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS2 , ΔmepM1 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 4: growth +
MepS2/MepM2	PF1573	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS2 , ΔmepM2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 8: no growth
MepS1/MepS2/MepM2	PF1575	$\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepS1 , ΔmepS2 , ΔmepM2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$	Row 9: no growth

[0458] **Conclusion:** MepM1 deficiency restores growth to Prc-deficient *Pseudomonas* strains.

Deficiency in any one or more of MepS1, MepS2, and MepM2 activity fails to restore growth to a *Pseudomonas* Prc-deficient mutant. Unexpectedly, in the presence of a MepS1 deficiency the growth advantage conferred by the MepM1 deficiency is eliminated. To restore high cell density growth to Prc-deficient *P. fluorescens*, it is necessary to inactivate MepM1 while leaving a functional copy of MepS1.

[0459] Example 3. Optimal conditions for robust growth and Fab' production by recombinant host strains

[0460] Based on the combinatorial knockout data, two host strains having different protease knockouts and containing the anti-TNF-alpha Fab' expression plasmid, p688-48, were generated and tested for growth and recombinant protein production under varying induction conditions including two temperatures (25 deg C and 32 deg C), pH (6 and 7.2), and induction OD575 (60 and 120). **Table 7** shows pH, temperature, induction OD575, and Fab' titers at 24, 48, 72, and 120 hours post induction for each of the two strains, STR87639 ($\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepM1 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$) + p688-048 and STR87640 ($\Delta\text{prc1}\Delta\text{prc2}$, ΔhslUV , ΔmepM1 , ΔmepS2 , ΔpyrF , $\text{lsc}::\text{lacIQ1}$) + p688-048. Samples analyzed were prepared from soluble fractions of whole fermentation broth harvested at 24, 48 and 72 hours post induction. Soluble fractions were prepared by sonication of diluted whole fermentation, followed by centrifugation to

separate soluble and insoluble fractions. At 120 hours post induction, samples analyzed were prepared from cell free culture supernatant (cell free broth) following centrifugation of whole broth to separate the cell pellet and cell free broth. Titer of assembled Fab measured using non-reducing SDS-CGE analysis, using the system internal mass ladder.

[0461] Table 7. Growth condition study data

Ferm. Run No.	Strain ID	pH	Temp (C)	Induction OD575	Titer at I24 (mg/L)	Titer at I48 (mg/L)	Titer at I72 (mg/L)	Titer at I120 (mg/L)
1	STR87639	7.2	25	60	0	0	0	0
2	STR87639	6	25	120	0	0	147	230
3	STR87639	6	32	60	0	549	414	272
4	STR87639	7.2	32	120	0	0	46	12
5	STR87640	7.2	25	60	0	0	0	55
6	STR87640	6	25	120	0	0	0	96
7	STR87640	6	32	60	176	237	204	254
8	STR87640	7.2	32	120	0	0	0	70

[0462] I24: 24 hours post induction; I48: 48 hours post induction, I72: 72 hours post induction, I120: 120 hours post induction. Samples analyzed prepared from whole broth at 24, 48 and 72 hours post induction. Samples analyzed prepared from cell free culture supernatant (cell free broth) 120 hours post induction. Titer = assembled Fab measured using non-reducing SDS-CGE analysis.

[0463] The STR87639 lysate was analyzed by LC-MS and found to contain several species in the non-reduced intact mass, including HC / LC / LC dimer, and fragments of both HC and LC, and assemblies that include HC and LC fragments. The correct Fab mass (47,761) was observed in low abundance.

[0464] Conclusion: Both STR87639 and STR87640 produced the highest Fab' yields at pH 6.0, 32 deg C, and when induced at an OD575 of 60. STR87639 produced the highest yield, but accumulated proteolytic fragments of the Fab' heavy and light chains.

[0465] Example 4. Optimization of host strains for recombinant protein production I: identification of additional proteases that influence recombinant protein quality

[0466] Identification of proteases

[0467] To identify other factors that influence proteolysis of the anti-TNF-alpha Fab' fragment, two intermediate strains were created by introducing plasmid p688-048 containing the open reading frame of the anti-TNF-alpha Fab' heavy chain and light chain, and a co-transcriptional *P. fluorescens* DsbC protein disulfide isomerase on host backgrounds DC1032: Δprc1, Δprc2, ΔhslUV, ΔpyrF, lsc::lacI^{Q1} (creating STR36036), and DC867: Δprc1Δprc2, MepM1(P410L), ΔpyrF, lsc::lacI^{Q1} (creating PF1558). Biological replicates were grown in duplicate and sampled along the growth curve representing the initial culture seeded at low cell density, mid log phase growth, time post induction (I) at 12hr, and 48hr. The samples were processed for whole transcriptomic RNA sequencing at Genewiz (San Diego, CA). Quality trimmed Fastq files were returned and processed using the open sourced software STAR aligner compiled from source code and run with the following parameters: outSAMtype BAM SortedByCoordinate and quantMode GeneCounts. (See, e.g., Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013 Jan 1;29(1):15-21. doi: 10.1093/bioinformatics/bts635. Epub 2012 Oct 25. PMID: 23104886; PMCID: PMC3530905, incorporated herein by reference.) Quantified genes were annotated into a data frame using the open sourced featurecounts software compiled from source code (as described by Liao Y, Smyth GK and Shi W. featureCounts: an efficient general-purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 30(7):923-30, 2014, incorporated herein by reference) and the data was normalized and fitted to a linear regression model with interactions. Statistical significance of gene counts was done by calculating pValues based on the Wald test of the mean normalized gene counts between STR36306 (functional MepM1) and PF1558 (deficient MepM1). Genes impacting upregulation were visualized by volcano plot by plotting statistical significance (pValue) versus log2fold change of the mean normalized transcript counts cumulatively over time and between seed and I12. Time points used for comparison were taken from samples exhibiting the greatest change via a principal component analysis. The results are shown in **Tables 8 and 9**.

[0468] **Table 8. RNAseq comparing DC1032 host with MepM1-deficient host**

Gene	GeneID	log2fold change*	SEQ ID NO
PROKKA01104	Serralysin precursor; extracellular alkaline metalloprotease (RXF04495.2; PROKKA_01104)	0.61382	9
PROKKA01141	Protease HtpX (RXF05137; PROKKA_01141) amino acid	1.7	39

PROKKA02909	Protease Murein L,D transpeptidase (RXF01911; PROKKA_02909) amino acid	1.11	41
PROKKA01390	DegP2	-0.423888 (p=0.18)	31

[0469] *Cumulative difference across all fermentation time up to I12; p value < 0.05.

[0470] **Table 9. Transcription over fermentation time in MepM1-deficient host**

Gene	GeneID	log2fold change*	SEQ ID NO
PROKKA01104	Serralysin precursor; extracellular alkaline metalloprotease (RXF04495.2; PROKKA_01104)	3.01	9
PROKKA01141	Protease HtpX (RXF05137; PROKKA_01141) amino acid	4.96	39
PROKKA02909	Protease Murein L,D transpeptidase (RXF01911; PROKKA_02909) amino acid	2.0	41
PROKKA01390	DegP2	-0.783 (p=0.01)	31

[0471] *I12 vs. Seed; p value < 0.05.

[0472] **Conclusion:** This comparative analysis showed that extracellular alkaline metalloprotease RXF04495.2 belonging to the matrix metalloprotease class EC 3.4.24.40 (SEQ ID NO: 9) is upregulated both cumulatively and temporally across fermentation time in a Prc-deficient host cell having a MepM1 deficiency when compared with a Prc-deficient MepM1+ host cell.

[0473] **Example 5. Optimization of host strains for recombinant protein production II: introduction of additional protease mutations and strain evaluation**

[0474] **Extracellular alkaline metalloprotease RXF04495.2 deficiency**

[0475] Using a *P. fluorescens* host of genotype Δ prc1, Δ prc2, Δ hslUV, Δ mepM1, an RXF04495.2-deficient host was made by a method similar to that used to generate the combinatorial knockouts of the genes described in Example 2, in this case using a RXF04495.2 homology arm (SEQ ID NO: 61). Colony PCR verified knockouts were used to create the PF1596 host background having the following genotype: Δ prc1, Δ prc2, Δ hslUV, Δ mepM1, Δ RXF04495.2 metalloprotease, Δ pyrF, Δ lsc::lacIQ1. Resulting host PF1596 was then transformed with plasmid pFNX7800 containing the Fab' only expression cassette encoding the Fab' heavy chain operably linked to the Slmt secretion signal (SEQ ID NO: 25) and the Fab' light chain operably linked to the Azu secretion signal (SEQ ID NO: 26). These heavy and light chains were

expressed co-transcriptionally, in a variety of host backgrounds under the control of a tac promoter for IPTG inducible expression.

[0476] Further protease deficiencies and overexpressed proteins

[0477] DegP2 was selected as a further protease to inactivate and test in host strains for recombinant protein expression. However, despite screening more than 2000 clones, no DegP2 inactivation was obtained in the candidate protease-deficient host backgrounds. As an alternative approach, a catalytically dead DegP2 protease (DegP2 S219A, SEQ ID NO: 29) that outcompetes the endogenous protease was expressed, either on a separate plasmid under the control of the mannitol promoter (Pmtl), on the same plasmid under control of Pmtl, or co-transcriptionally with the Fab' HC and LC. Folding modulator PDIA6 (SEQ ID NO: 27) was expressed co-transcriptionally.

[0478] Evaluation of Protease-Deficient Host Strains

[0479] The anti-TNF-alpha Fab' produced by the recombinant host strains described in **Table 10** was evaluated for light chain proteolysis and/or heavy chain clipping.

[0480] Table 10. Recombinant protease-deficient host strains

	Strain ID	Host ID	Genotype	Plasmid and Encoded Amino Acid Sequences*
1	STR92557	PF1559	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ pyrF, lsc::lacI ^{Q1}	pFNX7420: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26) (genes co-transcribed from Ptac), DegP2S219A (SEQ ID NO: 29) (gene transcribed from Pmtl), PyrF
2	STR87639	PF1559	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ pyrF, lsc::lacI ^{Q1}	P688-048: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DsbC (SEQ ID NO: 60) (genes co-transcribed from Ptac), PyrF
3	STR92567	PF1559	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ pyrF, lsc::lacI ^{Q1}	pFNX7422: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), PDIA6 (SEQ ID NO: 27) (genes all co-transcribed from Ptac), PyrF
4	STR94974	PF1596	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ RXF04495.2; metalloendopeptidase, Δ pyrF lsc::lacI ^{Q1}	pFNX7800: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26) (genes co-transcribed from Ptac), PyrF
5	STR94975	PF1596	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ RXF04495.2;	pFNX7420: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26) (genes co-transcribed from Ptac)

			metalloendopeptidase, Δ pyrF, lsc::lacI ^{Q1}	DegP2S219A (SEQ ID NO: 29) (transcribed from Pmtl), PyrF
6	STR94976	PF1596	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ RXF04495.2; metalloendopeptidase, Δ pyrF, lsc::lacI ^{Q1}	pFNX7421: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DegP2S219A (SEQ ID NO: 29) (genes co-transcribed from Ptac), PyrF
7	STR94977	PF1596	Δ prc1 Δ prc2, Δ hslUV, Δ mepM1, Δ RXF04495.2; metalloendopeptidase, Δ pyrF, lsc::lacI ^{Q1}	pFNX7422: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), PDIA6 (SEQ ID NO: 27) (genes co-transcribed from Ptac), PyrF

[0481] *In each plasmid, the HC (heavy chain) and LC (light chain) amino acid sequences include N-terminally fused secretion signals Slmt (SEQ ID NO: 11) and Azu (SEQ ID NO: 13), respectively, as annotated in **Table 14** for SEQ ID NOS: 25 and 26. Corresponding nucleic acid sequences used are as shown in **Table 14** (SEQ ID NO: 25 is a fusion of SEQ ID NOS: 11 and 21, encoded by SEQ ID NOS: 12 and 22, respectively; SEQ ID NO: 26 is a fusion of SEQ ID NOS: 13 and 23, encoded by SEQ ID NOS: 14 and 24, respectively).

[0482] The host strains in **Table 10** above were cultured and induced according to the design shown in **Table 11** below.

[0483] Table 11. Induction Design

Unit	Harvest at	Strain ID	Actual Induction OD
DG3_u1	I24	STR92557	105
DG3_u2	I24	STR92557 mtl promoter	96.8
DG3_u3	I36	STR92557	100
DG3_u4	I48	STR92557	108.6
DG3_u5	I24	STR94973	97.6
DG3_u6	I36	STR94973	83
DG3_u7	I48	STR94973	87.4
DG3_u8	I24	STR94974	105.8
DG4_u1	I36	STR94974	87.6
DG4_u2	I48	STR94974	97.4
DG4_u3	I24	STR94975	110.4
DG4_u4	I36	STR94975	106.8

DG4_u5	I48	STR94975	103
DG4_u6	I24	STR94976	78.8
DG4_u7	I36	STR94976	87
DG4_u8	I48	STR94976	93.8

[0484] Strains were grown at pH 6.0, 32 deg C, in mineral salts medium with glycerol as a carbon source induced at the OD₅₇₅ as shown, and harvested at 24, 36, or 48 hours after induction. The harvested cells were suspended to 25% solids in 75 mM phosphate, 100 mM sodium chloride, pH 7. The cell suspension was then lysed/homogenized and centrifuged at 15,000 x g for 30 minutes. Clarified lysate was then loaded onto a Protein L resin pre-equilibrated with 75 mM phosphate, 100 mM NaCl, pH 7.4. The affinity captured Fab' was washed with 3 column volumes (CV's) of 50mM BisTris, 1M NaCl, pH 7.0 followed by 3 CV's of 50mM Tris, pH 7.2. The Fab' was eluted with 3-10 CV's of elution buffer (75mM acetate, pH 3.4) and immediately neutralized to pH 7-8 with 2.4 M Tris base. Purity of the Fab' was assessed with reduced and non-reduced SDS-CGE.

[0485] *Light chain proteolysis in protease-deficient host strains*

[0486] Proteolysis of the Fab' fragment was quantitated by enriching the Fab' using capto-L affinity chromatography and subsequent imaging and quantification by NR-SDS-CGE of the enriched fragments. The results are shown in **Fig. 6**. Strains were grown and harvested at the post-induction times (in hours) as shown on the y-axis, and the recombinant protein was Capto-L enriched and analyzed by NR-SDS-CGE. The x-axis shows the percent of proteolyzed light chain species in each lane. A brief summary of the strains tested is shown in **Table 12** below.

[0487] Table 12. Summary of recombinant protease-deficient host strains

Strain ID	Host ID	Secretion Signal (HC/LC)	Deficient Protease Activities	Co-overexpressed Folding Modulator
STR92557	PF1559	Slmt/Azu	Prc1/Prc2/hslUV/MepM1	DegP2S219A
STR87639	PF1559	Slmt/Azu	Prc1/Prc2/hslUV/MepM1	DsbC
STR92567	PF1559	Slmt/Azu	Prc1/Prc2/hslUV/MepM1	PDIA6 (co-transcriptional with Fab')
STR94974	PF1596	Slmt/Azu	Prc1/Prc2/hslUV/MepM1/ Metalloprotease	---
STR94975	PF1596	Slmt/Azu	Prc1/Prc2/hslUV/MepM1/ Metalloprotease	DegP2S219A

STR94976	PF1596	Slmt/Azu	Prc1/Prc2/hslUV /MepM1/ Metalloprotease	DegP2S219A (co- transcriptional with Fab')
STR94977	PF1596	Slmt/Azu	Prc1/Prc2/hslUV /MepM1/ Metalloprotease	PDIA6 (co- transcriptional with Fab')

[0488] As shown by **Fig. 6**, the recombinant Fab' light chain produced by STR94974, which is deficient in MepM1 and the RXF04495.2 metalloprotease, was least proteolyzed. Proteolysis was significantly decreased at I24 and I36. At I24 virtually no proteolysis was observed in backgrounds containing deletion of RXF04495.2. The Fab' expression in PF1596 shows a further decrease of proteolysis by half at I36. The RXF04495.2 deficiency dramatically reduced the degradation of the light chain (LC) of the Fab.

[0489] *Titer of recombinant Fab' produced by protease-deficient host strains*

[0490] The anti-TNF-alpha Fab' sample titers were obtained via biolayer interferometry using the Octet Red 96 system, measuring TNF- α binding activity. Data were collected in Data Acquisition software version 11.0. All experiments were performed in 1X kinetics buffer (Sartorius, part number 18-1105). High-precision streptavidin biosensors (Sartorius, part number 18-5117) were incubated with 115 nM biotinylated TNF- α (Acro Biosystem, part number TNA-H8211) for 60 seconds followed by incubation in assay buffer for 60 seconds to establish a baseline. The binding of the samples was then measured to detect active Fab'. The results for the expression strains that produced the highest titers of active Fab' are summarized below.

[0491] **Fig. 7** shows that in host strains STR94974, STR94975, and STR94977, the anti-TNF-alpha Fab' fragment was produced at titers up to 1g/L at 24 hrs and 4g/L at 72 hrs, as assessed by NR-SDS-CGE or by biolayer interferometry (BLI) with immobilized TNF-alpha. Host cell line PF1596 can also be transformed with an expression cassette containing a fusion of the DsbA and Azu secretion signals to the heavy and light chain amino acid sequences, respectively, which can result in Fab' titers up to 1 g/L at the 2L scale in MepM KO prc null lines by NR-CGE analysis (data not shown).

[0492] *Growth comparison of protease-deficient host strains*

[0493] **Figs. 8A and 8B** compare growth of STR87639, which is Prc and MepM1 deficient, with STR92557, STR92567, STR94974, and STR94976 for 24 and 48 hours after induction, respectively. Growth phase was conducted at 32 deg C, pH 6.5, and the induction target was OD575=80. The cells were induced with IPTG and production phase was conducted at 32 deg C, pH 6.5 for either 24 or 48 hours. The overexpression of various folding modulators did not appear to impact growth significantly, nor did stacking of the metalloprotease deletion (STR94974 and STR94976). With the exception of STR87639 all strains continued to increase in cell density for a period of time post induction

[0494] **Fig. 9** compares growth of *P. fluorescens* Prc-deficient Fab' expression strains STR87639, STR92473, STR94994, STR94995, and STR94996, and STR94998 (see **Table 13**). Growth phase was conducted at 32 deg C, pH 6.5. Induction target OD575 was 100, carried out at 32 deg, pH 6 (STR87639, STR92473), or was 80, carried out at 32 deg, pH 6.5 (others).

[0495] Lines, from top to bottom:

[0496] Closed circles, solid line: STR87639 (MepM1 deficient)

[0497] Triangles, solid line: STR92473 (MepM1 and MepM2 deficient)

[0498] Diamonds, solid line: STR94995 (MepS2 deficient)

[0499] Circles, dashed line: STR94998 (not MepM or MepS deficient; no expression construct)

[0500] Open circles, solid line: STR94996 (MepS1 and MepS2 deficient)

[0501] Squares, solid line: STR94994 (MepS1 deficient)

[0502] The results showed that deficiency in MepS1, MepS2, or both MepS1 and MepS2, failed to restore growth in Prc-deficient host cells. Deficiency in MepM1 or both MepM1 and MepM2, restored growth in Prc-deficient host cells.

[0503] **Table 13. Recombinant protease-deficient host strains used in Fig. 9 growth comparison**

	Strain ID	Host ID	Genotype	Plasmid and Encoded Amino Acid Sequences*
1	STR87639	PF1559	Δ prc1 Δ prc2, Δ hslUV, ΔmepM1 , Δ pyrF, lsc::lacI ^{Q1}	P688-048: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DsbC (SEQ ID NO: 60) all co-transcribed from Ptac, PyrF
2	STR92473	PF1584	Δ prc1 Δ prc2, Δ hslUV, ΔmepM1 , ΔmepM2 , Δ pyrF, lsc::lacI ^{Q1}	P688-048: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DsbC (SEQ ID NO: 60) all co-transcribed from Ptac, PyrF
3	STR94995	PF1582	Δ prc1, Δ prc2, Δ hslUV, ΔmepS2 , Δ pyrF, lsc::lacIQ1	P688-048: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DsbC (SEQ ID NO: 60) all co-transcribed from Ptac, PyrF
4	STR94998	DC1032	Δ prc1, Δ prc2, Δ hslUV, Δ pyrF, lsc::lacIQ1	pDOW1169: empty expression vector
5	STR94996	PF1562	Δ prc1, Δ prc2, Δ hslUV, ΔmepS1 , ΔmepS2 , Δ pyrF, lsc::lacIQ1	P688-048: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DsbC (SEQ ID NO: 60) all co-transcribed from Ptac, PyrF

6	STR94994	PF1583	Δ prc1, Δ prc2, Δ hslUV, Δ mepS1, Δ pyrF, lsc::lacIQ1	P688-048: Fab' HC (SEQ ID NO: 25), LC (SEQ ID NO: 26), DsbC (SEQ ID NO: 60) all co-transcribed from Ptac, PyrF
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[0504] *In each plasmid, the HC (heavy chain) and LC (light chain) amino acid sequences include N-terminally fused secretion signals Slmt (SEQ ID NO: 11) and Azu (SEQ ID NO: 13), respectively, as annotated in **Table 14** for SEQ ID NOS: 25 and 26. Corresponding nucleic acid sequences used are as shown in **Table 14** (SEQ ID NO: 25 is a fusion of SEQ ID NOS: 11 and 21, encoded by SEQ ID NOS: 12 and 22, respectively; SEQ ID NO: 26 is a fusion of SEQ ID NOS: 13 and 23, encoded by SEQ ID NOS: 14 and 24, respectively).

Table 14. Table of Sequences

SEQ ID NO	Name	Sequence*
1	MepM1 amino acid RXF01291 <i>P. fluorescens</i>	MTTEPSKAPPLYPKTHLLAASGIAALLSLALLVFPSSDVEAKRT SLSLDLESPVEQLTQDQDASDAQQATNTATESPFAQIESTPEDT QQAAQEAPAAKSPQHREVIVGKGDTLSTLFEKVGLPAAAVNDV LASDKQAKQFTQLKRGQKLEFELTPDGQLNNLYTSISDLESISL SKGAKGFANRITTKPVMRSAYVHGVINSSLSQSAARAGLSHSM TMDMASVFGYDIDFAQDIRQGDEFDVIYEQKVANGKVVGTGNIL SARFTNRGKTYTAVRYTNKQGNSSYYTADGNSMRKAFIRTPVDF ARISSRFSMGRKHPILNKIRAHKGVDAAPRGTPIKAAGDGKVL LAGRRGGYGNTVIIQHGNTYRTLYGHMQGFAKGVKTGGNVKQGQ VIGYIGTTGLSTGPHLHYEFQVNGVHVDPLGQKLPMADPIAKAE RARFMQQSQPLMARMDQERSTLLASAKR
2	MepM1 example nucleic acid encoding SEQ ID NO: 1	ATGACCACTGAACCGTCTAAAGCGCCGCCGCTTTACCCGAAGAC CCACCTGCTCGCCGCAAGTGGTATCGCCGCCCTTCTCAGCCTGG CACTGCTGGTATTCCCTTCCAGTGACGTTGAAGCCAAACGAACA TCCCTGAGCCTTGATCTGGAAAGCCCAGTTGAACAACTGACACA AGATCAAGACGCTTCCGACGCTCAACAAGCCACAAACACTGCAA CTGAATCACCTTTTCGCCAGATCGAAAGCACACCCGAAGACACC CAGCAAGCCGCCAGGAAGCACCTGCAGCAGCCAAGAGTCCCCA GCATCGCGAAGTCATCGTGGGCAAAGGCGACACACTCTCGACCC TGTTTCGAAAAAGTTGGGTGCCTGCCGCCGCTGTAAATGACGTG CTCGCCAGCGATAAGCAAGCCAAGCAATTCCTCAGCTCAAACG

		TGGTCAAAGCTTGAATTTGAGCTGACGCCAGACGGCCAGTTGA ACAACCTGTACACCAGCATCAGTGACTTGGAAAGCATCAGCCTG AGCAAAGGCGCCAAAGGCTTCGCATTCAACAGAATCACCACCAA ACCCGTCATGCGTTCCGCCTACGTACATGGCGTGATCAACAGCT CCCTGTTCGCAGTCGGCCGCGCGTGCGGGCCTGTCGCATAGCATG ACCATGGACATGGCCAGCGTATTTGGCTACGACATCGACTTCGC CCAGGACATCCGTCAAGGCGACGAATTCGACGTGATCTACGAAC AGAAAGTAGCCAACGGAAGTGGTCGGCACTGGCAACATTCTT TCTGCACGCTTACAAACCGTGGCAAAACCTACACCGCCGTGCG CTACACCAACAAACAAGGCAACAGCAGCTACTACACGGCTGATG GCAACAGCATGCGTAAGGCCTTCATCCGTACACCCGTTGACTTT GCCCCGATTAGCTCGCGTTTCTCCATGGGCCGCAAGCATCCAAT TCTGAACAAAATTTCGCGCACACAAGGGCGTCGACTATGCCGCGC CGCGTGGCACGCCAATCAAAGCAGCGGGCGACGGCAAGGTCTTG TTGGCGGGGCGCCGTGGTGGTTACGGCAATACGGTGATCATCCA GCACGGCAACACTTACCGCACGCTGTACGGCCACATGCAAGGGT TCGCCAAGGGCGTCAAGACAGGCGGCAACGTGAAACAGGGCCAA GTGATCGGCTACATCGGTACCACCGGCCTCTCCACCGGCCCGCA CTTGCACTACGAGTTCCAGGTCAACGGCGTACACGTGACCCAT TGGGCCAGAAGCTGCCGATGGCCGACCCGATTGCCAAGGCCGAA CGCGCGCGCTTCATGCAACAGAGCCAGCCGCTGATGGCACGGAT GGATCAAGAGCGCTCCACCTTGCTGGCTTCGGCGAAGCGTTAA
3	MepM2 amino acid RXF03916 <i>P. fluorescens</i>	MPRLLSLLMLLCLTFNAHDSYITRTL NKPVPGGVAVVELGPSA TAPKATYQGKPVLVVKEQDNWLAIVGIPLTVKPGNERISSGGRN LPFIVGYKKYPEQRITLKNKSQVNPDP AQLKRIEGELAVQLKAY RSFSPNLPSNLVLDKPVNGPLSSKFGVRRFFNGEERNPHSGLDF AVPAGTPIKTPANGKVILVGN YFFNGNTVFVDHGQGFI SMFCHM SKIDVRVGQQLVRGAVVGKVGSTGRATGPHMHNVS LNDARVDP AIFIGAFQP
4	MepM2 example nucleic acid encoding SEQ ID NO: 3	ATGCCACGCCTACTGAGCCTGTTGATGCTGTTGTGCCTCACGTT TAACGCCCACGCCGACAGCTACATCACGGAACCTGAACAAAC CCGTGCCTGGCGGCGTGCCGTCGTGCGAACTAGGCCCTTCGGCC ACAGCGCCGAAAGCCACCTACCAGGGCAAGCCGGTGCTGGTGGT CAAGGAGCAGGACAACCTGGCTGGCGATTGTGCGGCATCCCGTTGA CGGTCAAGCCTGGCAACGAGCGCATCAGCAGCGGGGGGCGCAAC

		CTGCCGTTTATCGTCGGCTACAAGAAGTATCCGGAACAACGCAT CACCTTGAAGAACAAAAGCCAGGTCAACCCCGACCCGGCCAGC TCAAGCGCATCGAAGGCGAATTGGCAGTGCAGCTCAAGGCTTAC CGCAGCTTCAGCCCGAATTTGCCGAGCAATCTGGTGCTGGATAA ACCGGTGAACGGGCCGCTGTCTGAGCAAGTTCGGGGTGCGACGCT TCTTCAACGGCGAAGAGCGCAACCCGCACTCGGGCTGGACTTC GCCGTACCGGCCGGCAGACCGGATCAAGACACCCGCCAATGGCAA GGTGATTCTGGTCGGCAATTACTTCTTCAACGGCAATACCGTGT TTGTGACCATGGCCAGGGGTTTATCAGCATGTTCTGCCATATG TCGAAGATCGATGTGAGGGTGGGTGAGCAACTGGTGCGCGGTGC GGTAGTCGGCAAAGTAGGCTCGACAGGCCGGGCCACTGGGCCGC ATATGCACTGGAACGTCAGCCTGAACGATGCACGGGTAGATCCG GCGATTTTTATCGGCGCGTTTCAACCCCTGA
5	MepS1 amino acid RXF04923 <i>P. fluorescens</i>	MLNRFAPLVPLALVTLFLGCASHPQQVAEQQKPVQVQNAKFVAA QSASVYEEEVATEKELAEFSDSKPYQLPLLADSLERGMSLIGT RYRFGGTSEAGFDCSGFIGYLFREEAGMNLPRSTREMINVNAPL VARNNLKPGDLLFFSTSGRGRVSHAGIYLGDNQFIHSSRRSGG VRVDNLGDSYWSKTFIEAKRALAMAPTTVTASK
6	MepS1 example nucleic acid encoding SEQ ID NO: 5	ATGCTAAATCGCTTCGCACCCCTCGTGCCTCTCGCACTCGTTAC CCTGTTGTTTGGTTGCGCCTCCACCCCTCAGCAGGTGGCAGAAC AGCAAAAACACAGGTTCAAAATCAGGCAAAGTTCGTTGCTGCA CAGTCTGCTTCTGTTTATGAAGAAGAGGTGGCAACCGAAAAAGA ACTCGCCGAGTTCTCCGACAGCAAGCCTTACCAGCTGCCACTTC TGGCCGACAGCATCCTTGAGCGCGGCATGTCCTTGATCGGTACC CGTTACCGTTTCGGCGGCACCTCGGAAGCCGGTTTTGATTGCAG CGGTTTCATTGGCTACCTGTTTCGTGAAGAAGCCGGTATGAACC TGCCGCGCTCCACGCGGAGATGATCAACGTGAATGCACCGTTG GTCGCACGAAACAACCTCAAGCCCGGTGATCTGCTTTTCTTTAG TACCAGTGCCGCGGTCTGTGTCAGCCACGCCGGTATCTACCTGG GCGATAACCAGTTTATTTCATTCCAGCAGCCCGCAGTGTTGGT GTTCCGGTCGATAACCTCGGTGACAGCTACTGGAGCAAAACCTT CATCGAAGCCAAGCGCGCACTCGCCATGGCCCCGACGACGGTTA CCGCTAGTAAGTAA
7	MepS2 amino acid <i>P. fluorescens</i>	MSTSARLMLIVCAALLSACASRTPPPAPVAVKPKPVFNATQNF SPAAEDVLFRLGLVGTPYRWGNTPDSGFDCSLIGFVFRDAA

		GISLPRTTRELIVMRAQDVSEQNLQTDLLFFATGGGSRVSHAG IYVGEGRFVHAPQTGGTVKLDLTLKAYWQNAYLSAKRVLPGNLA RNP
8	MepS2 example nucleic acid encoding SEQ ID NO: 7	ATGTCGACCTCGGCCCCGCTGATGCTTATTGTTTGCGCCGCGCT GCTCAGCGCCTGCGCCAGTCGCACACCGCCGCCCGCGCCCGTCG CGGTCAAGCCTAAGCCGGTGTTCAACTATGCCACCCAGAAATTC TCGCCAGCTGCCGAAGACGTGCTCTTTTCGTGCGCTGGGCCTGGT CGGCACGCCTTATCGCTGGGGCGGCAACACACCGGACTCGGGTT TTGATTGCAGCGCCTGATCGGCTTTGTATTCCGCGACGCTGCT GGCATCTCATTGCCGCGCACCCCGTGAACTGATCGTGATGCG TGCCAGGACGTGAGCGAACAACCTGCAGACCGGCGACCTGC TGTTCTTCGCCACCGGTGGTGGTTCGCGGGTCAGCCATGCGGGT ATTTATGTGGGGGAGGGGCGCTTCGTACACGCGCCGCAAACCGG CGGTACGGTGAAGCTGGATACGCTATCCAAAGCGTATTGGCAGA ATGCCTACCTGAGTGCCAAACGCGTGTTCAGGGAATCTGGCG CGTAACCCCTGA
9	Serralysin precursor; extracellular alkaline metalloprotease amino acid (RXF04495.2; PROKKA_01104) <i>P. fluorescens</i>	MHIPVRQSSYSRPSDKLQPDLSPEHQVVLWANNKKSFTTDQAA KHITRGGFKFHDRNNDGKIVVGYNFAGGFNAAQKERARQALQYW ADVANIEFVENGPNTDGTISIKGVPGSAGVAGLPNKYNSNVQAN IGTQGGQNPMAGSHFLGLLIHELGH TLGLSHPGKYDGQGFNYDR AAEYAQDTKARSVMSYWTETHQPGHNFAGRSPGAPMDDIAAAQ RLYGANTKTRNTDTTYGFNSNSGREAYSLKQGS DKPIFTVWDGG GNDTLDFSGFTQNQTINLKAESFSDVGGLRGNVSIAGVSVENA IGGTGNDTLTGNEGNRLTGKGADKLHGGAGADTFVYRRASDS TPQAPDIIQDFQSGSDKIDLTGVVQEAGLKSLSFVEKFSGKAGE AVLGQDAKTGRFTLAVDITGNGTADLLVASQSQIKQADVIWNGQ APTVTPTPEPTVVPVSDPVPTPTSEPTPEPTPEPAPLPVPTPR PGGGFIGKIFSSFKGFIKKVWSIFR
10	RXF04495.2 example nucleic acid encoding sequence SEQ ID NO: 9	ATGCATATCCCTGTTAGGCAGTCTTCTTACTCGCGTCCTTCAGA TAAGTTACAGCCCGATCTTTCACCCGATGAACACCAAGTTGTTT TCTGGGCCAACATAAAAAATCTTTCACCACGGATCAGGCCGCG AAACACATCACCCGCGGTGGCTTCAAGTTTCATGATCGCAACAA TGATGGAAAAATCGTCGTGGGTTATAACTTTGCGGGCGGCTTCA ATGCGGCTCAGAAAGAACGGGCCAGGCAAGCCCTTCAGTACTGG GCGGATGTTGCTAATATCGAATTTGTTGAAAAATGGCCCGAACAC

		GGATGGCACAATAAGCATCAAGGGTGTTCGGGGTTCGGCAGGCG TCGCGGGGTTCGCCAACAAATATAATTTCGAACGTCCAGGCCAAT ATAGGCACCCAGGGTGGGCAAAACCCGGCGATGGGCAGTCACTT CCTGGGCTTATTGATCCATGAACTGGGGCATACCCTGGGGCTGA GTCATCCAGGTAAATACGACGGCCAGGGTTTCAATTACGATCGG GCTGCCGAATATGCCCAGGACACCAAGGCTCGCAGTGTTCATGAG CTATTGGACGGAGACTCATCAGCCGGGGCACAATTTTGCCGGGC GCAGCCCGGGTGCCCCGATGATGGACGATATCGCCGCCGCCAG CGGCTCTACGGCGCCAACACCAAAACCCGGAATACCGACACCAC CTACGGCTTCAATTCCAATTCAGGCCGGGAGGCTTATAGCCTCA AGCAGGGGAGCGACAAGCCGATCTTCACCGTCTGGGACGGTGA GGTAATGACACGCTCGACTTCTCCGGGTTCACCCAGAACCAAAC CATCAACCTCAAGGCTGAGTCATTCTCGGACGTGGGGGGCTTGC GAGGAAATGTGTCGATTGCCAAGGGTGTGAGTGTGGAACGCC ATTGGCGGTACAGGCAACGATACCTTGACGGGGAACGAGGGGCAA CAATCGGCTCACGGGCGGCAAGGGGGCCGATAAGCTGCACGGCG GAGCTGGAGCAGACACGTTTGTGTTACCGCCGCGCCAGCGATTCA ACGCCGCAGGCACCGGACATCATCCAGGACTTCCAGAGCGGGAG CGACAAGATCGACCTGACCGGTGTTGTTTCAGGAGGCGGGGCTCA AGTCGCTGAGCTTCGTCGAGAAATTCAGCGGCAAGGCGGGCGAG GCCGTGCTCGGCCAAGACGCGAAAACCGGCCGTTTCACGTTGGC GGTGGACACAACGGGAAATGGTACGGCGGATCTACTGGTTGCCA GCCAAAGCCAGATCAAACAGGCGGATGTGATCTGGAACGGTCAG GCGCCGACAGTGACGCCAACGCCTGAACCCACTGTGGTGCCTGT GTCAGATCCCGTGCCGACCCCTACTTCAGAGCCGACTGAACCTG AACCACGCCTGAGCCCGCCCCCTTTGCCCGTCCCGACTCCACGG CCTGGAGGAGGGTTTATCGGGAAAAATTTTTTCATCATTCAGGG GTTTCATAAAAAAAGTGTGGTCGATATTCAGGTGA
11	Slmt secretion signal amino acid <i>P. fluorescens</i>	MRSRLFNFLSCLLLSATAVQSAQA
12	Slmt example nucleic acid encoding SEQ ID NO: 11	ATGCGCAGTCGCCTTTTCAACTTTTTATCTTGCTGCTTCTTTC CGCCACTGCCGTTCAATCCGCCCCAG

13	Azu secretion signal amino acid <i>P. fluorescens</i>	MFAKLVAVSLLTLASGQLLA
14	Azu example nucleic acid encoding SEQ ID NO: 13	ATGTTTGCCAACTCGTTGCTGTTTCCCTGCTGACTCTGGCGAG CGGCCAGTTGCTT
15	DsbA secretion signal amino acid <i>P. fluorescens</i>	MRNLILSAALVTASLFGMTAQA
16	DsbA example nucleic acid encoding SEQ ID NO: 15	ATGCGTAATCTGATCCTCAGCGCCGCTCTCGTCACTGCCAGCCT CTTCGGCATGACCGCACAA
17	AnsB secretion signal amino acid <i>P. fluorescens</i>	MKSALKNVIPGALALLLLFPVAAQA
18	AnsB example nucleic acid encoding SEQ ID NO: 17	ATGAAATCTGCATTGAAGAACGTTATTCCGGGCGCCCTGGCCCT TCTGCTGCTATTCCCCGTCGCCGCCAGGCC
19	PorE secretion signal amino acid <i>P. fluorescens</i>	MKKSTLAVAVTLGAIAQQAGA
20	PorE example nucleic acid encoding SEQ ID NO: 19	ATGAAGAAGTCCACCTTGGCTGTGGCTGTAACGTTGGGCGCAAT CGCCCAGCAAGCAGGCGCC
21	Anti-TNF-alpha Fab' heavy chain amino acid (certolizumab)	EVQLVESGGGLVQPGGSLRLSCAASGYVFTDYGMNWRQAPGKG LEWMGWINTYIGEPYADSVKGRFTFSLDTSKSTAYLQMNSLRA EDTAVYYCARGYRSYAMDYWGQGLTVTVSSASTKGPSVFPLAPS SKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ SSGLYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS CDKTHTCAA

22	Anti-TNF-alpha Fab' heavy chain example nucleic acid encoding SEQ ID NO: 21	GAAGTGCAACTGGTGGAGAGCGGCGGTGGCTTGGTTCAGCCGGG TGGCTCCCTGCGTCTGTCTGTGCGGCCCTCCGGGTACGTGTTCA CCGACTACGGCATGAACTGGGTCCGCCAGGCCCCAGGGAAGGGT CTGGAATGGATGGGCTGGATCAACACGTATATCGGCGAACCAGT TTATGCGGACAGCGTAAAAGGGCGCTTCACCTTTAGCTTGGATA CCTCCAAAAGTACGGCCTACCTGCAGATGAATTCCCTGCGGGCA GAGGATACCGCGGTGTATTACTGCGCTCGCGGCTACCGCAGCTA CGCGATGGACTACTGGGGCCAAGGCACCCTGGTGACGGTGAGTT CGGCCAGCACCAAGGGCCCTAGCGTGTTCCTACTCGCCCCCAGC AGCAAATCGACCTCGGGCGGTACGGCCGCACTCGGCTGCCTGGT GAAGGACTATTTCCCGAGCCGGTGACCGTCAGTTGGAACAGTG GTGCCCTGACTAGCGGCGTGACACCTTTCCCGCCGTTCTGCAG AGCTCGGGCTTGTACTCCTTGTCTCGTCCGTCTGTAAGTGTGCCCAG CAGCTCGCTCGGCACCCAGACCTACATCTGCAATGTCAACCACA AGCCGAGCAACACCAAAGTGGATAAGAAGGTGGAACCGAAGTCC TGCGACAAGACCCATACCTGTGCGGCC
23	Anti-TNF-alpha Fab' light chain amino acid (certolizumab)	DIQMTQSPSSLSASVGRVTITCKASQNVGTNVAWYQQKPGKAP KALIYSASFLYSGVPYRFSGSGSGTDFTLTISLQPEDFATYYC QQYNIYPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
24	Anti-TNF-alpha Fab' light chain example nucleic acid encoding SEQ ID NO: 23	GACATTTCAGATGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGT AGGGGACCGCGTGACCATCACCTGTAAAGCCAGTCAAAACGTCTG GTACCAACGTGGCATGGTATCAACAAAAACCGGGTAAAGCCCCC AAAGCGTTGATCTACTCCGCCAGTTTCTGTATAGCGGCGTGCC GTACCGCTTCAGCGGCTCCGGCAGCGGTACCGACTTTACCCTGA CCATTTCCTCGCTGCAACCCGAGGACTTTGCGACCTACTATTGC CAGCAGTATAACATCTACCCGCTGACGTTTCGGGCAGGGCACGAA GGTCGAAATCAAACGGACCGTAGCGGCACCGAGTGTGTTCATCT TCCCTCCGAGCGACGAACAGTTGAAGTCCGGCACCGCCTCGGTC GTGTGCCTGCTCAATAACTTCTACCCACGCGAGGCTAAGGTGCA ATGGAAGGTGGACAACGCCCTGCAGTCGGGCAATAGTCAGGAAT CGGTGACTGAACAGGATTCCAAGGATAGCACCTACTCGCTCAGC AGCACGCTGACCTTGTCGAAGGCCGATTACGAGAAGCATAAGGT

		CTACGCGTGCGAAGTGACGCACCAGGGCCTGTCTCGCCGGTTA CTAAGAGCTTTTAACCGTGGCGAGTGC
25	Slmt leader (underlined text) fused to anti-TNF- alpha Fab' heavy chain amino acid	<u>MRSRLFNFLSCLLLSATAVQSAQAEVQLVESGGGLVQPGGSLRL</u> SCAASGYVFTDYGMNWVRQAPGKGLEWMGWINTYIGEPYADSV KGRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCARGYRSYAMDYW GQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCAA
26	Azu leader (underlined text) fused to anti-TNF- alpha Fab' light chain amino acid	<u>MFAKLVAVSLTLASGQLLADIQMTQSPSSLSASVGDRTITCK</u> ASQNVGTNVAWYQQKPGKAPKALIYSASFLYSGVPYRFSGSGSG TDFTLTISLQPEDFATYYCQQYNIYPLTFGQGTKVEIKRTVAA PSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS GNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
27	PDIA6 folding modulator amino acid (Q15084) human PDI isoform 6	LYSSDDVIELTPSNFNREVIQSDSLWLVEFYAPWCGHCQRLTP EWKKAATALKDVKVKGAVDADKHHSLSGGQYGVQGFPTIKIFGSN KNRPEDYQGGRTGEAIVDAALSALRQLVKDRLGGRSGGYSSGKQ GRSDSSSKKDVIELTDDSFDKNVLDSQVWVVEFYAPWCGHCCKN LEPEWAAAASEVKEQTKGKVKLA AVDATVNQVLASRYGIRGFPT IKIFQKGESPVVDYDGGRTSRDIVSRALDLFSDNAPPPELLEIIN EDIAKRTCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKY KKKMWGLWTEAGAQSELETALGIGGFGYPAMAAINARKMKFAL LKGSFSEQGINEFLRELSFGRGSTAPVGGGAFTIVEREPWDGR DGELPVEDDIDLSDVELDDLKDEL
28	PorE leader (underlined text) fused to PDIA6 folding modulator amino acid	<u>MKKSTLAVAVTLGAIAQQAGALYSSDDVIELTPSNFNREVIQ</u> DSLWLVEFYAPWCGHCQRLTPEWKKAAATALKDVKVKGAVDADKH HSLGGQYGVQGFPTIKIFGSNKNRPEDYQGGRTGEAIVDAALSA LRQLVKDRLGGRSGGYSSGKQGRSDSSSKKDVIELTDDSFDKNV LDSEDVWVVEFYAPWCGHCCKNLEPEWAAAASEVKEQTKGKVKLA AVDATVNQVLASRYGIRGFPTIKIFQKGESPVVDYDGGRTSRDIV SRALDLFSDNAPPPELLEIINEDIAKRTCEEHQLCVVAVLPHIL DTGAAGRNSYLEVLLKLADKYKKKMWGLWTEAGAQSELETALG IGGFGYPAMAAINARKMKFALLKGSFSEQGINEFLRELSFGRGS TAPVGGGAFTIVEREPWDGRDGELPVEDDIDLSDVELDDLKDEL EL

29	DegP2 (S219A) amino acid <i>P. fluorescens</i> (Do family serine endopeptidase; NCBI Reference Sequence WP_198833397.1 with S219A included as indicated by bold text)	MSIPRLKSYLSIVATVVLVGLQALPAQAVELPDFTQLVEQASPAV VNISTTQKLPDRKVSNNQMPDLEGLPMLREFFERGMPPQPRSPR GGGGQREAOQLGSGFIISPDGYILTNHVIADADEILVRLADRS ELKAKLIGTDPRSDVALLKIEGKDLFVLKLGKSQDLKAGQWVVA IGSPFGFDHTVTQGI VSAIGRSLPNENYVPFIQT DVPINPGNAG GPLFNLAGEVVGINSQIYTRSGGFMGVSFAIPIDVAMDVSNQLK SGGKVSRLGVLGVVIEVKNDLAESFGLDKPAGALVAQIQDNGPA AKGGLKVGDVILSMNGQPIIMSADLPHLVGALKAGGKAKLEVIR DGKRQNVELTVGAIPPEGATLDALGNAKPGAERSSNRLGI AVVE LTAEQKKTFDLQSGVVIKEVQDGPAAALIGLQPGDVI THLNNQAI DTTKEFADIAKALPKNRSVSMRVLRQGRASFITFKLAE
30	DegP2 (S219A) example nucleic acid encoding SEQ ID NO: 29	ATGTCGATAACCACGTTTGAAGTCTTACTTATCCATAGTCGCCAC AGTGCTGGTGCTGGGTCAGGCCTTACCTGCGCAAGCGGTCGAGT TGCCTGACTTCACCCAACTGGTGGAGCAGGCCTCGCCTGCCGTG GTGAACATCAGTACCACGCAGAAGCTGCCGGATCGCAAAGTCTC GAACCAGCAGATGCCCCGACCTGGAAGGCTTGCCGCCCATGCTGC GCGAGTTCTTCGAACGAGGGATGCCGCAACCACGCTCCCCCGT GGCGGCGGTGGCCAGCGCGAAGCCCAATCCCTGGGCTCCGGCTT CATCATTTTCGCTGACGGCTATATCCTCACCAACAACCACGTGA TTGCCGATGCCGACGAGATTCTCGTGCGCCTGGCCGACCGCAGT GAACTCAAGGCCAAGCTGATTGGCACCGATCCACGTTCGGACGT GGCCTTGCTTAAATCGAGGGCAAGGACTTGCCGGTGCTTAAGC TGGGCAAGTCCCAGGACCTGAAGGCCGTCAGTGGGTGGTTCGCG ATCGGTTTCGCGTTTCGGCTTTGACCACACCGTTACCCAAGGCAT CGTCAGCGCCATCGGTTCGAGCCTGCCGAACGAAAACCTACGTAC CGTTCATCCAGACCGACGTGCCGATCAACCCGGGTAAACGCCGGT GGCCCGCTGTTCAACCTGGCCGGCGAAGTGGTGGGGATCAACTC GCAGATCTACACCCGCTCCGGCGGCTTCATGGGCGTGTCTTTTCG CGATCCCAATCGATGTGGCCATGGACGTCTCCAATCAGCTCAAA AGCGGCGGCAAGGTCAGCCGCGGCTGGTTGGGCGTGGTAATCCA GGAAGTGAACAAGGACCTGGCTGAGTCCTTCGGTCTCGACAAGC CGGCCGGTGCCCTGGTTGCGCAGATTGAGGACAATGGCCCTGCG GCCAAAGGCGGCTGAAAGTCGGTGACGTCATCTGAGCATGAA CGGCCAGCCGATCATCATGTGCGCAGACTTGCCCTCATTTGGTCG

		GCGCGCTCAAGGCCGGCGGCAAAGCCAAGCTGGAAGTGATTTCGT GATGGCAAGCGCCAGAACGTCGAACTGACCGTAGGTGCCATCCC GGAAGAAGGCGCGACCCCTGGATGCCCTGGGCAACGCCAAGCCCCG GTGCCGAGCGCAGCAGTAACCGCCTGGGTATCGCCGTGGTTGAA CTGACCGCCGAGCAGAAGAAAACCTTCGACCTGCAAAGCGGTGT GGTGATCAAGGAAGTTCAGGACGGCCAGCCGCTTGATCGGCC TGCAACCGGGTGACGTGATCACTCACTTGAACAACCAGGCAATC GATACCACCAAGGAATTGCGCCGACATCGCCAAGGCGTTGCCGAA GAATCGCTCGGTGTCGATGCGCGTCCTGCGTCAAGGCCGTGCCA GCTTCATTACCTTCAAGCTGGCTGAG
31	DegP2 amino acid (Protease RXF07210; PROKKA_01390) <i>P. fluorescens</i> (Do family serine endopeptidase; NCBI Reference Sequence WP_198833397.1) (leader underlined at 1-27; DegP2 protein 28-478; catalytic triad amino acids with/without leader at H116/89, D120/93, S219/192, in bold)	<u>MSIPRLKSYLSIVATVVLGQALPAQAVELPDFTQLVEQASPAV</u> VNISTTQKLPRKVSNQMPDLEGLPPMLREFFERGMPPRSPR GGGGQREASLGSFGFIISPDGYILTNNHVIADADEILVRLADRS ELKAKLIGTDPRSDVALLKIEGKDLVVLKLGKSQDLKAGQWVVA IGSPFGDHTVTQGI¹VSAIGRSLPNENYVPFIQTDVPINPGNSG GPLENLAGEVVGINSQIYTRSGGFMGVSFAIPIDVAMDVSNQLK SGGKVSRLGVLGVVIEVNKDLAESFGLDKPAGALVAQIQDNGPA AKGGLKVGDVILSMNGQPIIMSADLPHLVGALKAGGKAKLEVIR DGKRQNVELTVGAIPEEGATLDALGNAKPGAERSSNRLGIAVVE LTAEQKKTFDLQSGVVIKEVQDGPAALIGLQPGDVITHLNNQAI DTTKEFADIAKALPKNRSVSMRVLRQGRASFITFKLAE
32	MepS (Spr) amino acid <i>E. coli</i> (UniProtKB - P0AFV4; EC 3.4.17.13; U.S. Pat.	MVKSQPILRYILRGIPATAVAVLLSACSANNTAKNMHPETRAVG SETSSLQASQDEFENLVRNVDVKSRLMDQYADWKGVRYRLGGST KKGIDCSGFVQRTFREQFGLELPRSTYEQQEMGKSVSRNLRTG DLVLFRASTGRHVGIYIGNNQFVHASTSSGVIISSMNEPYWKK RYNEARRVLSRS

	No. 9,493,559, incorporated herein by reference)	
33	Prc1 amino acid <i>P. fluorescens</i>	MKHLFPSTALAFFIGLGFASMSTNTFAANSWDNLQPDRDEVIAS LNVVELLKRHHYSKPPLDDARSVIIYDSYLKLLDPSRSYFLASD IAEFDKWKTKQFDDFLKSGDLQPGFTIYKRYLDRVKARLDFALGE LNKGVDKLDFTQKETLLVDRKDAPWLSTAAALDDLWRKRVKDEV LRLKIAGKEPKAIQELLTKRYKNQLARLDQTRAEDIFQAYINTF AMSYDPHTNYLSPDNAENFDINMSLSLEGIGAVLQSDNDQVKIV RLVPAGPADKTKQVAPADKIIIGVAQADKEMVDVVGWRLDEVVKL IRGPKGSVVRLEVIPHTNAPNDQTSKIVSITREAVKLEDQAVQK KVLNLKQDGKDYKLGVIEIPAFYLDKFAFRAGDPDYKSTTRDVK KILTELQKEKVDGVVIDLRNNGGSLQEATELTSLFIDKGPTVL VRNADGRVDVLEDENPGAFYKGPMLLVNRLSASASEIFAGAMQ DYHRALIIGGQTFGKGTVQTIQPLNHGELKLTLAKFYRVSGQST QHQQVLPDIDFPSIIDTKEIGESALPEAMPWDTIRPAIKPASDP FKPFLAQLKADHDTRSADAEFVFIRDKLALAKKLMEEKTVSLN EADRRAQHSSIENQQVLVLENTRRKAKGEDPLKELKKEDEDALPT EADKTKPEDDAYLAETGRILLDYLKITKQVAKQ
34	Prc1 example nucleic acid encoding SEQ ID NO: 33	ATGAAGCATCTGTTCCCCAGCACCGCCCTCGCTTTTTTCATTGG TCTCGGCTTCGCGTCGATGTGACCAATACGTTTCGACGCCAATA GCTGGGACAACCTTCAGCCTGATCGCGATGAGGTGATTGCCAGC CTTAACGTCGTCGAGTTGCTTAAGCGCCATCACTACAGCAAGCC GCCGCTGGACGACGCTCGCTCAGTGATCATCTACGACAGCTACC TCAAGCTGCTGGACCCGTCGCGCAGCTACTTCCTGGCCAGCGAT ATCGCTGAGTTCGACAAGTGGAAGACGCAATTCGACGACTTCCT CAAGAGCGGCGACCTGCAGCCTGGCTTACCATCTACAAGCGCT ACCTAGACCGCGTCAAAGCGCTCTGGACTTCGCCCTGGGTGAG CTGAACAAAGGCGTCGACAAGCTCGATTTACCCAGAAAGAAAC CCTTCTGGTGGACCGCAAGGACGCCCCCTTGGCTGACCAGCACCG CAGCCCTAGACGACCTGTGGCGCAAACGCGTCAAGGACGAAGTG CTGCGCTTGAAGATCGCCGGCAAAGAGCCCAAGGCCATTCAAGA GCTGTTGACCAAGCGCTACAAAAACCAGCTGGCGCGCCTGGACC AGACCCGTGCCGAGGATATCTTCCAGGCCTACATCAACACCTTT GCGATGTCCTACGACCCGCACACCAATTATCTGTCGCCAGATAA

		CGCGGAAAATTTGATATCAATATGAGTCTGTCCCTGGAAGGCA TCGGTGCCGTCTTGCAAAGCGACAATGACCAGGTGAAGATTGTA CGTCTGGTGCCGGCAGGCCCGGCTGACAAAACCAAGCAAGTGGC ACCGGCCGACAAGATCATCGGCGTGGCCAGGCCGACAAAGAGA TGGTCGATGTGGTCTGGCTGGCGCCTGGACGAAGTGGTCAAGCTG ATCCGTGGGCCTAAAGGCAGCGTGGTGGCCTGGAAGTGATTCC GCACACCAATGCACCGAACGACCAGACCAGCAAGATCGTGTCCA TCACCCGTGAAGCGGTGAAGCTCGAAGACCAGGCCGTGCAGAAG AAAGTCCTCAACCTCAAGCAGGATGGCAAGGACTACAAGCTGGG GGTGATTGAAATCCCGGCCTTCTACCTGGACTTCAAGGCGTTCC GTGCCGGTGATCCGGACTACAAGTCCACCACCCGCGACGTGAAG AAAATCCTCACAGAACTGCAGAAAGAGAAAGTCGACGGCGTGGT CATCGACCTGCGCAACAACGGCGGCGGCTCCCTGCAGGAAGCCA CCGAGCTGACCAGCCTGTTTATCGACAAGGGCCCGACCGTGTTG GTACGCAACGCTGACGGCCGTGTGACGTGCTCGAAGACGAGAA CCCGGGGGCCTTCTACAAAGGGCCGATGGCGCTGCTGGTCAACC GCCTCTCGGCCTCGGCCTCGGAGATTTTCGCCGGTGCCATGCAG GACTACCACCGTGCCTGATCATCGGCGGCCAGACCTTCGGCAA AGGCACCGTGACAGCATCCAGCCGCTGAACCATGGCGAGCTTA AGCTGACACTGGCCAAGTTCTACCGGGTCTCCGGGCAGAGCACC CAGCATCAGGGCGTACTGCCGGATATCGATTTCCCGTCGATCAT CGACACCAAGGAAATTGGCGAAAGCGCCCTGCCTGAAGCCATGC CGTGGGACACCATCCGCCCTGCGATCAAGCCGGCGTGGATCCG TTCAAGCCGTTCTTGGCACAGCTGAAGGCTGACCACGACACCCG CTCTGCCAAGGATGCCGAGTTCGTGTTTATCCGCGACAAGCTGG CCCTGGCCAAGAAGCTGATGGAAGAGAAGACCGTCAGCCTCAAC GAAGCGGATCGCCGTGCACAGCACTCCAGCATCGAGAATCAGCA ACTGGTGCTGGAAAACACCCGCCGCAAGGCCAAAGGTGAAGACC CGCTCAAAGAGCTGAAGAAAGAAGATGAAGACGCGCTGCCGACC GAGGCGGATAAAACCAAGCCGGAAGACGACGCCTACTTGCCGA GACTGGCCGGATCCTGCTGGATTACCTGAAGATCACCAAGCAGG TGGCCAAGCAGTAA
35	Prc2 amino acid <i>P. fluorescens</i>	MLHLSRLTSLALTIALVIGAPLAFADQAAPAPATAATTKAPLP LDELRTFAEVMDRIKAAYVEPVDDKALLENAIKGMLSNLDPHSA YLGPEDFAEHQESTSGEFGLGIEVGSSEDGQIKVVSPIDDTPAS

		KAGIQAGDLIVKINGQPTRGQTMTEAVDKMRGKLGQKITLTLVR DGGNPFVDVTLARATITVKS VKSQLLESYGYIRITQFQVKTGDE VAKALAKLRKDNGKKLNGIVL DLRNPNP GGVLQSAVEVVDHFVTK GLIVYTKGRIANSELRF SATGNDLSENVPLAVLINGGSASASEI VAGALQDLKRGVLMGTTSFGKGSVQTVLPLNNERALKITTALYY TPNGRSIQAQGIVPDIEVRRAKITNEIDGEYYKEADLQGH LGNG NGGADQPTGSRAKAKPMPQDDDYQLAQALSLLKGLSITRSR
36	Prc2 example nucleic acid encoding SEQ ID NO: 35	ATGCTGCATTTGTCCCGCCTCACTTCGCTGGCCCTGACGATCGC CCTGGTGATCGGCGCGCCTCTGGCTTTTGCCGACCAGGCCGCAC CGGCTGCACCCGCCACGGCTGCGACGACCAAGGCCCATTTGCCG CTGGACGAGCTGCGTACCTTTGCCGAGGT CATGGACCGGATCAA GGCAGCGTATGTCTGAACCCGTAGACGACAAGGCCCTGCTGGAAA ATGCCATCAAGGGCATGCTCAGCAACCTCGACCCGCACTCCGCC TACCTGGGCCCCGGAAGATTTGCCGAGCTGCAGGAAAGCACCAG CGGTGAGTTTCGGCGGCCTGGGCATCGAAGTGGGCTCCGAAGACG GCCAGATCAAAGTGGTCTCGCCTATCGACGACACCCCGGCGTCC AAGGCCGGTATCCAGGCCGGCGACCTGATCGTGAAGATCAACGG CCAGCCAACCCGCGGCCAGACCATGACCGAAGCCGTCGACAAGA TGCGCGGCAAGCTCGGCCAGAAGATCACCCCTGACCCTGGTACGC GACGGCGGCAACCCGTTTGACGTGACCCTGGCCCGCGCGACCAT CACGGTCAAGAGCGTGAAAAGCCAGCTGCTGGAGTCGGGGTACG GTTATATCCGTATCACCCAGTTCCAGGTCAAGACCGGCGACGAA GTGGCCAAGGCCCTGGCCAAGCTGCGCAAAGACAACGGCAAGAA GCTCAACGGCATCGTGCTTGACCTGCGCAACAACCCAGGCGGCG TGTTGCAGTCGGCGGTGAGGTGGTCGACCACTTCGTCACCAAG GGCCTGATCGTCTACACCAAGGGCCGTATCGCCAACTCAGAGTT GCGCTTCTCGGCCACCGGCAACGACCTCAGCGAGAACGTGCCAC TGGCGGTATTGATCAACGGTGGCAGCGCCTCGGCTTCGGAAATC GTCGCGGTGCCCTGCAAGACCTCAAGCGCGGCGTGCTGATGGG CACCACCAGCTTCGGCAAAGGCTCGGTGCGACCGTATTGCCGC TGAACAACGAGCGTGCGCTGAAGATCACACGGCGCTGTACTAC ACGCCCAACGGCCGCTCGATCCAGGCCAGGGCATCGTGCCGGA CATCGAAGTACGCCGCGCCAAGATCACCAACGAGATCGACGGCG AATACTACAAAGAGGCCGACCTGCAAGGTACCTGGGCAATGGC AACGGCGGTGCCGACCAGCCAACCGGCAGCCGCGCCAAGGCCAA

		GCCGATGCCGCAGGACGATGACTACCAACTGGCCCAGGCACTCA GCCTGCTCAAGGGCTTGAGCATCACCCGCAGCCGTTGA
37	HslU amino acid (RXF01957; PROKKA_01919) <i>P. fluorescens</i>	MSMTPREIVHELNRHIIGQDDAKRAVAIALRNRWRRMQLPEELR VEVTPKNILMIGPTGVGKTEIARRLAKLANAPFIKVEATKFTEV GYVGRDVESIIRDLADAALKMLREQEVTKVSHRAEDAAEERILD ALLPPARMGFNEDAAPATDSNTRQLFRKRLREGQLDDKEIEIEV AEVSGVDISAPPGMEEMTSQLQNLFANMGKGKKKSRKLKVKEAL KLVRDEEAGRLVNEEELKAKALEAVEQHGVFIDEIDKVAKRGN SGGVDVSRREGVQRDLLPLIEGCTVNTKLGMVKTDHILFIASGAF HLSKPSDLVPELQGRLPPIRVELKALTPGDFERILSEPHASLTEQ YRELLKTEGLGIEFQADGIKRLAEIAWQVNEKTENIGARRLHTL LERLLEEVSFSAGDMAGAQNGEAIKIDADYVNSHLGELAQNEDL SRYIL
38	HslV amino acid (RXF01961; PROKKA_01920) <i>P. fluorescens</i>	MTTIVSVRRHGKVVMMGGDGQVSLGNTVMKGNNAKKVRRLYHGQVL AGFAGATADAFITLFEFEGQLEKHQGHVRAAAVELAKEWRTDRS LSRLEAMLAVANKDASLIITGNGDVVEPEHGLIAMSGGGGYAQA AASALLKKTDLISAREIVETALGIAGDICTVFTNHNQTIIEEQDLAE
39	Protease HtpX amino acid (RXF05137; PROKKA_01141) <i>P. fluorescens</i>	MMRILLFLATNLAVVLIASVTLISLFGFNGFMAANGVDNLNLQLL IFCAVFGFAGSLFSLFISKWMAKMSTSTQIITQPRTRHEQWLMQ TVEQLSQEAGIKMPEVGIFFAYEANAFAFGWNKNDAVAVSQGL LERFSPDEVKAVLAHEIGHVANGDMVTLALVQGVVNTFVMFFAR IIGNFVDKVIIFKNEEGRGIAYFVATIFAELVLGFLASAIVMWFS RKREFRADEAGARLAGTSAMIGALQRLRSEQGLPVHMPDSLTA F GINGGIKQGLARLFMSHPLEERIDALRRRG
40	Protease HtpX example nucleic acid encoding SEQ ID NO: 39	ATGATGCGCATCCTGCTGTTCTTGCCCACTAACCTGGCGGTCGT ACTGATTGCCAGCGTCACCCTGAGCCTTTTGGCTTCAACGGGT TCATGGCGGCCAATGGGGTTGATCTGAACCTCAATCAGCTGCTG ATTTTCTGTGCGGTCTTTGGTTTTGCCGGCTCGCTGTTCTCGCT GTTTCATCTCCAAGTGGATGGCGAAGATGAGCACCAGCACCAGA TCATCACTCAACCCCGCACTCGCCATGAACAATGGCTGATGCAA ACCGTGAGCAGTTGTCTCAAGAAGCAGGCATCAAAATGCCCCGA AGTGGGGATTTTTCTGCTTATGAGGCCAACGCTTTGCCACCG GCTGGAACAAGAACGACGCACTGGTGGCTGTGAGCCAGGGCCTG CTGGAGCGGTTTTGCCCCGATGAAGTCAAGCGGTGCTGGCCCA CGAGATCGGCCACGTAGCCAACGGCGACATGGTCACCCTGGCAC

		TGGTACAGGGCGTGGTGAACACCTTCGTGATGTTCTTTGCGCGG ATCATCGGCAACTTTGTGACAAGGTCATCTTCAAGAACGAAGA AGGCCGTGGCATTGCCTACTTCGTGGCGACCATTTTCGCCGAGT TGGTCCTGGGCTTCCTGGCCAGCGCCATCGTGATGTGGTTCTCG CGCAAACGCGAGTTCCGCGCAGATGAAGCCGGCGCACGCCTGGC GGGCACCAGCGCAATGATCGGCGCGCTGCAACGCCTGCGCTCCG AACAGGGCCTGCCGGTGCATATGCCGGACAGCCTGACCGCCTTC GGCATCAACGGCGGCATCAAGCAGGGCCTGGCTCGCTTGTTCAT GAGCCACCCGCCGCTGGAAGAGCGGATTGACGCACTGCGTCGCC GGGGCTGA
41	Protease Murein L,D transpeptidase amino acid (RXF01911; PROKKA_02909) <i>P. fluorescens</i>	MFKKHACYLSICLLVAPLVATAETLPLEPLPVTTTPAPVALAPLQ QALAQLTSCPHLAPRIDAAALARLQTFYQQQGDAPLWAADERR QALHAQLMLLADDGLDPHTHSLPAVDATANVLCSDIANSQQYLQ ALQDLHYGRLQQSRFEPLWHSQPPSGDPNTEVLAFATGLHDMA QAFDQARPSADLYRSLRNAYAGVRQQPLPHWDPVAEGTLLRPGM NDPRVPELARRLHSGGYLAQLPSGNGKQYQGELVKAVKAFQLSH SLQADGVIGAGTVAELNISPAMRREQLRINLERFRWLAQDLEPE GVVVNVAQAQLSVYQSGIPVWQTRLQVGRAERQTPLLKSRITRL TLNPTWTIPPTIMREDKLPAILRLNPEYLRQQNLQVLDAEGHPLT PDQVDWARPGNILLRQQAGPRNPLGKIVMRFPNPYSVYLHDTPS QPLFTKGPRAFSSGCVRVEQPLLLRDLVTPAERTRTDELLATG ETHEFRLATPVPVLLGYWTVEVDROGELVYAPDIYARDPALIKA MGSVL
42	Protease Murein L,D transpeptidase example nucleic acid encoding SEQ ID NO: 41	ATGTTCAAAAAACACGCATGTTACTTGAGCATTTGCCTGCTCGT TGCACCATTTGGTCGCTACAGCCGAAACGCTGCCGTTAGAACCAC TGCCCGTCACCACCCCTGCACCGGTGCGCTCGCGCCGCTGCAA CAGGCCTTGGCGCAGTTGACCAGTGTCTGCCCCGACCTTGCGCC GCGTATCGATGCCGCCGCGTTGGCGCGCCTGCAAACCTTTTACC AGCAGCAGGGCGATGCCCCGCTATGGGCGGCTGACGAACGCCGG CAAGCCTTGATGCCAGTTGCTGATGCTTGCCGACGATGGCCT GGACCCACCCACTATAGCTTGCTGCGGTGGATGCCACGGCCA ACGTGCTGTGCAGCGATATCGCCAACAGCCAGCAGTACCTGCAA GCTCTGCAGGATTGCACTACGGGCGCCTGCAGCAATCGCGCTT TGAGCCCCCTTGGCATTCCCAGCCACCCAGTGGCGATCCGAATA CCGAGGTGCTGGCGTTGCCGCCACCGGCTGCACGACATGGCC

		CAAGCCTTCGATCAGGCCCGGCCAGCGCCGATTTGTACCGCAG CCTGCGCAATGCCTATGCCGGCGTGCGCCAGCAACCGCTGCCCC ATTGGGACCCGGTCGCCGAGGGCACGTTGTTGCGTCCTGGGATG AATGACCCTCGCGTGCCGGAAGTGGCGCGGCGCTGCACAGCGG CGGCTACCTGGCCCAGTTACCCAGCGGCAACGGCAAGCAGTACC AGGGCGAACTGGTCAAGGCGGTGAAAGCCTTCCAGCTCAGCCAC TCGTTGCAGGCCGACGGCGTGATCGGCGCCGGCACCGTGGCCGA ACTCAATATCAGCCCGCGATGCGTTCGTGAACAACTGCGCATCA ACCTCGAGCGTTTCCGCTGGCTGGCCAGGACCTGGAGCCTGAA GGCGTCGTGGTCAATGTGGCCGCCGCGCAACTGAGCGTGTACCA GAGCGGCATCCCAGTGTGGCAAACCCGCCTGCAAGTGGGCCGGG CCGAACGCCAGACGCCGTTGCTCAAGTCGCGCATCACCCGGCTG ACCCCTCAACCCACCTGGACCATCCCGCCGACCATCATGCGCGA GGACAACTGCCGGCCATCCGCCTCAACCTGAATACCTGCGCC AGCAAAACCTGCAAGTGCTCGACGCCGAAGGTCAACCGTTGACC CCCGACCAGGTGCGACTGGGCGCGCCCCGGCAATATCCTGCTGCG CCAGCAGGCCGGCCCGCGTAACCCGCTGGGCAAGATTGTGATGC GTTTCCCCAATCCGTATTCCGTATATCTGCACGACACCCCCAGC CAACCCCTTGTTACCAAGGGGCCGCGGGCGTTTCAAGTTCGGGATG CGTGCGGGTCGAGCAACCGTTGTTATTGCGCGACCTGCTGGTAA CGCCGGCCGAACGCACTCGCACCGATGAGCTGCTGGCGACCGGC GAAACCCATGAATTCAGGTTGGCCACGCCGGTACCGGTGCTGTT GGGGTATTGGACCGTGAAGTGGATCGCCAGGGCGAGCTGGTGT ACGCGCCGGATATTTATGCGCGTGACCCGGCGTTGATCAAGGCC ATGGGTAGCGTGTATAG
43	Protease/Autolytic Factor Hemolysin precursor amino acid (RXF09262; PROKKA_04435) <i>P. fluorescens</i>	MDVRQFAFLARQPSAALKRRDAFFGLPKRGLALILANALFWQPL LAQAEGIVVSAPGTTVGAAGNGVPVNIATPNGAGLSHNQFKDY NVGPNGVILNNGNGAMVNTQLGGIIVGNPNLKGGAAANVILNEVN GGSPSQLRGYTEVAGQSAKVIVANPYGVTCSGCGFINTPNVTLT TGKPVLDASGQLQRYEVDGGAVIDGQGLNASNVERFDIITRSA KINAQINARELNVIAGRNDVDAQSLKTTARADDGSAKPELAIDS SALGGMYAGAIKLVGTEAGVGKLDGTLAASGGDIQLDANGRLS MAQAAATGNVKVTAQNVDLTDKVYANGNVQVTSQAQALVNRKSLA AGQRIEINAASVNNPGIIIEAGVAADNSRNTTGDLVVNAQTVTTS GNLLASRALAITAAQALTNQGAI IQAKTVEVSSAKLTNQGASAR

		LFGEQSLAINSPAIVNLGGLIRFGEGQAATLNSASLDNRQGRIE MAGGSLVLTSAIDLNNSGGQVIANDLTVNAGNLNNQNGVLVAKTA TVTASNLDNSLKGLIQADGGALNLAVSNTFNNNQGFQAQASTDLN VTAGTLSSNAGGVLADTGKLTLTAAQQLNNAQGRLLQAGQGDI LHAANLDNQSGTIVGKQLLLDVAGGIDNRAGRVLGHDLDVRAS GLDNRNAGLLAGGAQGVSLLLKGPQQLNNAQGRLLQAGQGLQ ERFDNSAGILLGQTVDVTAQTFNNSNKGALVSDGGDVVFKVSDL LTNVGGQIDAGERSVLVKQLTTLNNDGGTLRGKRLDIAAQHLNN DNGQLLAGAEGLSYSGQDVSNRKGLILSGGALTELTNTRLDNQ GTVQGDSLTVTANNVDNCSGGLMASLVGNLQLTVEALANRGGKL FGKEQVTVSGASLDNSAGQISGNQINLTSRDLTNQGGGLVEANQ GLTLTGGLNLDNSANGQLRALGGASSRVNLSGALNNQNGTLEFGS QAFSLDAASLNNQNGMLQHAGTGLFHLNIASLEGSQGNIQMGMS ADWAFGKVDGLGRVQLNDVLTYSKSDQGLALQAGDRMASAKGLIL NVASLDNGGELLSDGDISITTGDI TNSGRVSALQTLTVAANNLS QNGGRLAATNARLTGGTLDNLGFLTARQQLDIAAAQINNRTL GAQGAVNLTAVNGITNAADTLLFSGGDMTLRSNGFSNSYGDVYS KGNLSFAARDGGRAVLFSNRSGTVESESGIGINAGFIENAKDEF ELGQTLTTGSLSWICGQHCGESDNWERGEITITYETYLEAATKDS VAARLVAGKNMMLQGD TVQNRYSLMAANGDLSITAGDLLNQGAA TRTGQRKLVIPTGPHVSDDLFERMQYVDVPAFNAATAAGNFDKA RFEELKSRSPNSLPFAYASDVTTWTNNSGPGYDATLQAGGTVNL NVARTLQNGTLHNNTLAQLTGTLGDDQTGIPVGGININLSKHAN DPSAQAPGSVLPVVG VAPGGGFVPVDYTGTAFAFPVDPPTSPTFQ LPKGEYGLFVKNA DPTSHYLIETNPEFTSVSGFFSSDYMLGKLG FTADNAWRRLGDGQYETRLIRDAVLAQTGQRFLAGGLYSADQF RYLMDNGLASKDALRLSLGVALTDQQVGALTHDIVWMENRVIEG QTVLVPVLYLAQADSRNVRGNSLIQGRDLNLVTGGDLINVGTLR ASNNLSAISSGSIYTGGLVEAGNNLSLLAQDSIRNAMAGEIRGK QVSLTALKGDI TNETTAIQVRD GAGMRTLTDTSAGTIVARENLA IDAGRDLTNRGALVAGNDANLTAGRDLNLIAASDTRVKHETRDG GEKSSITTDVKNLAASVTAGGNLNMQAGQDVNIIGSNATAGKDL NIAAGRDLNVASVSDMHNVEGKEKDGGKRIRTSDDQTTQVASVL TAGGDFVSQAGRDTTIVASMISAGNEAYLYSGDKLSLLAAENST HTLYDMKEKGSWAKKAQMDEVTRTTQVGTEIKTGGNLVLKSDG
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		DQLYQVAKLNSGKDIILDSGGAIVFEGVKDLHDESHTKSKSDLS WFSAGKKGNTDETLRQSELVAQGQLVIKAAEGIRIDVKQVDQQT VSQTV DAMVKADPNLAWLKQAEARGDIDWRQVKEIHESFKYDNS GLGAGAKIAIAIMMAAIMGPVGFGLQGATLAVSTSLSTTAVTST INNKGNLGAALKETVSANSLKSAAVAGFTAGALEYADTNWFAGA DGAGAGAGTSTSTVQGVTPSTGSTLAVTNSSKDI FTWTSAGDIA LRTGGRAVISSGISTAIQGGSGFDNFNAALLGEAGNVAMATGFN WVG DYVTFPNGSPQKIIAHALMGGLLAEATGSDFKTGAAAAGLN EALINQLVWAAQGNDDITLMLSQLTGLLAAAAVDGDLEKGSQIA QKATTFNYLYHEEVEEMLREVDSKTTEQEKREVRQRYAELDQQR NDEL DALCARDPQRCRGIATSLANDDQKLVDLVGRLRSQGQGGA ASAVGFVIGNNLDASSQIAADISSAGGGPLVKLGAEAIKAGVGI TLPSRSSSGKGKGSQVGAGSLEEAAGPKATGEVVPAPIVTSGA TRTG VVRTNAADWRALRNWDDLGYGQILSTENRAAIAKGRTPK VDDAWVKVFPEDAGLKGERIPMHVQGSPLTVPLPDTRHLDAHM PGGFRYNPGGPGSALPAYPPKKGAE
44	Protease/Autolytic Factor Hemolysin precursor example nucleic acid encoding SEQ ID NO: 43	ATGGATGTTGCGCAATTCGCCTTCCTGGCCCGCCAACCTTCTGC CGCCCTGAAGCGCCGGGACGCGTTCTTCGGCCTGCCAAGCGCG GGCTGGCCTTGATCCTTGCCAACGCACTGTTCTGGCAGCCGCTG CTGGCCAGGCCGAGGGCATTGTGGTCAGTGCGCCGGGCACCAC CGTGGGCGCGGCAGGCAATGGCGTGCCGGTGGTAAACATTGCCA CCCCAATGGCGCGGGCTTGTTCCATAACCAGTTCAAGGACTAC AACGTGCGCCCCAACGGCGTGATTCTCAACAATGGCAACGGCGC CATGGTCAACACCCAGCTGGGCGGGATCATCGTCGGCAACCCCA ACCTCAAGGGCGGCGCGGCGAACGTATCCTCAACGAAGTCAAC GGCGGCAGCCCCAGCCAGTTGCGCGGCTATACCGAAGTGGCGGG GCAGTCGGCCAAGGTATCGTGGCCAACCCGTACGGCGTGACGT GCAGCGGTTGCGGCTTTATCAACACCCCCAACGTACCCCTCACC ACCGGCAAACCGGTGCTCGACGCCAGCGGTCAATTGCAGCGCTA TGAAGTGGATGGCGGCGGGTGACCATCGACGGCCAAGGCTTGA ACGCCAGCAACGTGAACGCTTCGACATCATCACCCGCTCGGCC AAGATCAACGCACAAATCAACGCCCGGGAACCTCAACGTGATCGC CGGGCGCAACGACGTGATGCGCAAAGCCTGAAAACCACCGCCC GCGCCGATGACGGCAGCGCCAAGCCCGAGCTGGCGATCGACTCG TCGGCCCTGGGCGGCATGTATGCCGGCGGATCAAACCTGGTGGG

		CACCGAGGCCGGTGTGGGCGTGAAGCTCGACGGCACCCCTGGCCG CCAGTGGCGGCGATATTCAGCTCGACGCCAACGGGCGCCTGAGC ATGGCGCAGGCGGCGGCCACCGGTAACGTCAAGGTCACCGCGCA AAACGTCGACCTCACCGACAAGGTCTACGCCAACGGCAACGTGC AGGTCACCAGCGCCCAGGCTTTGGTCAACCGCAAGAGCATCGCC GCCGGCCAGCGCATCGAGATCAACGCGGCCAGCGTGAACAACCC CGGCATCATCGAAGCCGGCGTCGCCGCCGATAACAGCCGCAACA CCACGGGCGACCTGGTGGTGAACGCGCAAACCGTCACCACCAGC GGCAACCTGTTGGCCAGCCGCGCCCTGGCGATCACTGCCGCGCA AGCGCTGACCAACCAGGGCGCGATCATCCAGGCCAAGACCGTCG AGGTCAGCAGCGCCAACTCACCAACCAGGGCGCCAGCGCTCGC CTGTTTGGCGAGCAGAGCCTGGCGATCAACTCGCCGGCCATCGT CAACCTCGGCGGCTTGATCCGCTTCGGCGAAGGCCAGGCCGCCA CGCTCAACAGCGCCTCCCTGGACAACCGCCAAGGCCGTATCGAA ATGGCCGGTGGCAGCCTGGTGCTCACCAGTGCCGACCTGAACAA CAGCGGCGGGCAAGTCATCGCCAACGACCTGACCGTCAACGCCG GCAACCTGAACAACCAGAACGGCGTGCTGGTGGCCAAGACCGCG ACCGTCACTGCCAGCAACCTTGACAACAGCCTCAAGGGTTTGAT CCAGGCTGACGGTGGCGCGCTCAACCTCGCCGTTTCCAACACCT TCAACAACAACCAGGGTTTCGCCCAGGCCAGCACCGATCTGAAC GTTACGGCCGGCACCCCTCAGCAGCAACGCGAGGCGGCGTACTGAG CGCCGACACCGGCAAGCTCACCCCTCACCGCCGCACAACAACCTCA ACAACGCCCAGGGCCGCTTGACAGGCCGGGCAGGGCGATATCGAA CTGCACGCCGCGAACCTGGATAACCAGAGCGGCACGATCGTCGG CAAGCAACTGCTGCTCGACGTGGCCGGCGGCGACATCGACAACC GTGCCGGGCGCGTGTGGGTGACCACCTCGACGTGCGCGCCTCG GGCCTGGACAACCGCAACGCCGGCCTGCTGGCCGGTGGTGCCCA GGGCGTAAGCCTGCTGCTCAAAGGCCCGGGCCAGTTGCTCAACG CCCAGGGCCGCATCCAGAGCGAGGGCCTGCTGCAACTGCAAGGC GAGCGCTTCGACAACAGCGCCGGCATCTGCTGGGCCAGACCGT CGACGTGACCGCGCAGACCTTCAACAACAGCAACAAAGGCGCGC TGGTCAGCGATGGCGGTGATGTGGTGTTCAAGGTCAGCGACCTG CTCACCAACGTGGTGGCCAGATCGACGCGGGCGAACGCAGCGT GTTGGTCAAGCAGCTCACCAACCTCAACAACGACGGCGGCACCC TGCGCGGCAAGCGCCTGGACATCGCCGCCAGCACCTGAACAAC
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		GACAACGGCCAACTGCTGGCCGGCGCCGAAGGCCTGAGCTACAG CGGCCAGGATGTGAGCAACCGCAAGGGCCTGATCCTCAGCGGCG GCGCCCTCACC GAACTGAACACCACCCGCTGGATAATCAGGGC GGCACTGTGCAGGGCGACAGCCTGACCGTCACCGCCAACAACGT CGACAACGGCAGCGGGCGCCTGATGGCAAGCCTGGTCGGCAACC TGCAGCTCACTGTGGAAGCCCTGGCCAACCGTGGCGGCAAGCTG TTCGGCAAAGAACAAGTGACCGTCAGCGGCGCCAGCCTCGACAA CAGCGCGGGCCAGATCAGCGGCAATCAGATCAACCTGACCTCAC GCGACACGCTCACCAACCAGGGCGGTTTGGTTGAAGCCAACCAG GGCCTGACCCTCACTGGTGGCAACCTCGATAACAGCGCCAACGG CCAACTGCGTGCCCTGGGCGGCGCCAGCAGCCGCGTCAACCTCA GCGGTGCGTTGAACAACCAGAACGGCACCCCTCGAATTCGGTAGC CAGGCCTTCAGCCTTGACGCGGCCAGCCTCAACAACCAGAACGG GATGCTGCAACACGCCGGCACCGGCCTGTTCCACCTCAACATCG CCAGCCTCGAAGGCAGCCAGGGCAATATCCAGGGCATGGGCGAGC GCCGACTGGGCATTGCGCAAGGTCGACGGCCTGGGCCGCGTGCA ACTCAACGATGTGCTCACCTACAAGAGCGACCAAGGGCTGGCCC TCCAGGCCGGCGACCGCATGGCCAGCGCCAAGGGCTTGATCCTC AACGTGGCCAGCCTGGACAACGGCGGCGAACTGCTCAGCGACGG TGACATCAGCATCACCAACCGGCGATATCACCAACAGCGGCCGCG TCTCGGCCCTGCAAACACTCACCGTCGCCGCCAACCAACCTCAGC CAGAACGGCGGCCGCTGGCCGCAACCAATGCCCGCCTGACCCT GGGCGGCACCCTGGACAACCTCGGTTTCTCACCGCCCGCCAGC AACTGGACATCGCCGCCGCGCAAATCAACAACCGTGGCACCCCTC GGTGCCAGGGCGCAGTGAACCTCACGGCGGTCAACGGCATCAC CAACGCCGCCGACACGCTGCTGTTTACGGCGGCGGACATGACCC TGCGCAGCAATGGCTTCAGCAACAGCTATGGCGATGTCTACAGC AAAGGCAACCTGAGTTTTCGCCGCCCGGATGGCGGACGTGCCGT GCTGTTTACGAACCGCTCCGGCACCGTGGAAGCGAAGGCTCAA TTGGCATCAATGCAGGCTTTATCGAAAACGCCAAGACGAATTC GAACTCGGGCAGACACTGACCACCGGTAGCTTGAGCTGGATCTG TGGCCAGCACTGCGGCGAGAGCGACAACCTGGGAACGTGGCGAGA TCACCATCTACGAAACGTACCTCGAGGCGGCGACCAAGGACTCG GTAGCGGCGCGCCTGGTGGCGGGCAAAAACATGCTGCTGCAAGG CGACACGGTGAGAACCCTACAGCCTGATGGCCGCCAATGGCG
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		ACCTGAGCATCACTGCCGGAGACCTGCTCAACCAGGGCGCCGCC ACGCGCACGGGCCAGCGCAAGCTTGTCAATCGGCACGCCAGGTCA CGTCTCCGACGATTTGTTTGAACGCATGCAATATGTTGATGTGC CCGCGTTCAATGCCGGCCACGGCGGCTGGGAATTTGACAAAGGCG CGCTTCGAAGAACTCAAAAGCCGCTCACCCAATAGCCTGCCGTT CGCCTACGCCAGTGACGTCACCACCTGGACCAACAACAGCGGCC CCGGCTACGACGCCACCTGCAAGCGGGCGGCACGGTCAACCTC AACGTGCCCCGCACCCTGCAAAACGGCACGCTGCACAACAACAC CCTGGCCCAGTTGACCGGCACCCTCGGCGACGACCAGACCGGCA TCCCCGTGGCGGCATCAACATCAACCTGAGCAAAACAGCCAAAC GACCCGAGCGCCAGGCGCCCGGCAGTGTCTTGGCCGTGCTGGG CGTGGCCCCCTGGTGGCGGCTTCGTGCCCGTGGATTACACCGGCA CCGCGTTTGGCCCGGTGACCCCAACACCTCGCCACCTTCCAA CTGCCCAAGGGCGAATACGGCCTGTTCTGTCAAAAACGCCGACCC CACCAGCCACTACCTGATCGAGACCAACCCGAGTTACCTCGG TGTCGGGCTTCTTCAGCTCCGACTACATGCTCGGCAAAACCTCGGT TTCACCGCCGACAACGCCTGGCGCCGCTCGGTGACGGCCAGTA CGAAACCCGCCTGATCCGCGACGCCGTCTCGCGCAAACCGGCC AGCGCTTCCTCGCCGGCGGCCTGTACAGCGACGCCGACCAAGTTC CGCTACCTGATGGACAACGGCCTCGCCAGCAAAGACGCCCTGCG CCTGAGCCTGGGCGTGGCCCTCACCGACCAGCAAGTCGGCGGCC TGACCCACGACATCGTGTGGATGGAAAACCGCGTCATCGAAGGC CAGACCGTGCTCGTGCCGGTGCTGTACCTGGCCCAGGCCGACTC GCGCAACGTGCGCGGCAACAGCCTCATCCAGGGCCGCGACCTCA ACCTGGTCACCGGGCGGCGACCTGATCAACGTCGGCACCTTGC GCCAGCAACAACCTCTCCGCCATCAGTAGCGGCAGCATTTATAC CGGCGGCCTGGTCAAGCCGGCAACAATCTCAGCCTGCTGGCCC AGGACAGCATCCGCAACGCCATGGCCGGCGAAAATCCGCGGCAAG CAAGTCAGCCTCACGGCGCTCAAAGGCGATATCACCAACGAAAC CACCGCCATCCAGGTGCGTGACGGCGCCGGTATGCGCACCTCA CCGACACCAGCGCCGGCACCATCGTCGCCCGCGAAAACCTCGCC ATCGACGCTGGCCGCGACCTCACCAACCGAGGCGCGCTGGTAGC GGGCAACGACGCCAACCTCACCGCCGGCCGCGACCTCAACCTCA TCGCCGCCAGCGACACCCGCGTCAAACACGAGACCCGCGACGGC GGCGAGAAATCCAGCATCACCAACGACGTCAAAAACCTCGCCGC
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		CAGCGTCACGGCGGGCGGCAACCTCAACATGCAGGCCGGGCAAG ACGTCAACATCATCGGCAGCAATGCCACGGCCGGCAAAGACCTC AACATCGCCGCCGGCCGCGACCTCAACGTCGCCTCGGTGAGCGA CATGCACAACGTCGAGGGCAAGGAAAAGGACGGCAAAAAACGCA TCAGGACCTCGGACGACCAGACCACTCAAGTGGCAAGCGTGCTG ACGGCGGGTGGGGATTTTGTGAGCCAGGCGGGGCGTGATACCAC GATTGTGGCGAGCATGATCAGTGCGGGGAATGAGGCTTATCTGT ATAGCGGGGATAAGTTGAGTTTGTGTCGGCTGAGAACAGTACG CATACGTTGTATGACATGAAGGAGAAGGGAAGCTGGGGCGCTAA AAAGGCGCAGATGGATGAAGTGACCCGCACCACCCAGGTAGGGA CCGAGATCAAGACAGGTGGCAACCTAGTCCTTAAAGCGCAGGGC GACCAGCTGTATCAAGTTGCGAAGCTTAATAGCGGCAAGGACAT CATCCTTGATAGCGGTGGTGCAATTGTCTTTGAAGGCGTCAAGG ACCTGCACGATGAGAGCCACACTAAGAGCAAAAGCGACCTCTCG TGGTTGAGCGCTAAGGGCAAAGGTAATACAGACGAAACCTTGCG TCAGAGCGAGTTGGTTGCCCAAGGACAGCTTGTCATCAAGGCCG CCGAAGGCATTTCGTATCGACGTCAAACAGGTTCGATCAGCAGACT GTAAGCCAGACCGTTGATGCGATGGTCAAGGCTGATCCTAATTT GGCCTGGCTCAAGCAAGCTGAGGCACGTGGCGACATTGATTGGC GCCAGGTAAAGGAGATTACAGAGAGCTTCAAGTACGACAACCTCA GGGTGCGCGCCGGTGCCAAGATTGCGATTGCGATCATGATGGC GGCGATCATGGGGCCGGTAGGATTCGGGTTGCAGGGAGCCACCC TTGCGGTGAGCACCAGCCTGAGTACGACGGCAGTCACTAGCACC ATCAACAACAAAGGCAATTTGGGTGCAGCGCTTAAGGAAACGGT CAGCGCCAATAGCCTGAAAAGCGCAGCAGTCGCCGGGTTACAGG CGGGGGCTCTTGAGTATGCCGACACCAATTGGTTGCTGGTGCT GACGGTGCAGGTGCAGGTGCAGGCACAAGTACAAGCACAGTCCA AGGTGTTACCCCGAGTACGGGTTCACCTTGCGGTTACGAACT CCTCCAAAGATATTTTACCTGGACGTCAGCAGGCGATATCGCG CTGCGTACCGGTGGCCGGGCGGTAATCTCTAGCGGAATATCGAC GGCCATTCAAGGGGGAAGCTTCGGCGACAACCTCAATGCGGCCC TGTTGGGAGAGGCTGGCAACGTTGCAATGGCTACCGGTTTAAAT TGGGTGGGTGACTACGTACGTTCCCCAATGGCAGCCCTCAAAA GATTATTGCGCACGCTTTGATGGGGGGATTGCTGGCTGAAGCCA CAGGTAGCGATTTCAAACCGGGGCTGCCGCTGCCGGGCTGAAT
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		GAGGCACTCATCAATCAGTTGGTGTGGGCTGCTCAAGGCAATGA CGACATCACGCTGATGCTTTTACAGCTGACAGGCTTGTTAGCAG CTGCGGCGGTTCGATGGAGATTTGGAAAAAGGCTCTCAGATTGCT CAGAAGGCGACGACGTTCAACTATCTTTACCACGAAGAAGTCGA GGAAATGCTTCGGGAGGTAGATAGCAAGACTACGGAGCAAGAGA AGCGTGAGGTACGGCAGCGCTATGCGGAACCTTGATCAGCAGAGA AATGACGAGTTGGATGCGCTTTGCGCACGCGATCCGCAACGCTG CCGAGGTATTGCCACTTCCTTGCGGAACGATGATCAGAACTCG TTGATCTGGTAGGTAGGTGAGATCCCAAGGGCAGGGCGGTGCT GCTTCTGCGGTTGGTTTTGTGATAGGGAACAACCTAGACGCGTC CAGCCAAATTGCAGCAGATATCAGCTCTGCGGGCGGTGGGCCAT TAGTTAAGCTCGGTGCGGAGGCAATTAAGGCCGAGTTGGGATC ACACTGCCTTCACGTTCAAGCTCTGGTAAGGGGAAAGGAAGCCA AGTCGGCGCGGGTTCCTTGAAGAGGCGCGGGTCCAAAGGCGA CAGGCGAAGTAGTGCTCCCGCGCCTATTGTGACTTCTGGTGCG ACTAGGACAGGTGTTGTTCTGTACAAATGCCGAGATTGGAGAGC ACTGCGTAATAATTGGGATGACCTTGGGTATGGTCAAATCTTAA GTACTGAAAATCGGGCCGCGATTGCTAAAGGACGGACTCCAAAA GTCGACGATGCATGGGTAAAGGTTTTCTGAAGATGCAGGGCT AAAGGGCGAGAGAATTCCTATGCACCATGTTTCAGGGTTCGCCAC TTACTGTGCCACTGCCTGATACACGGCATTGAGTGCATATG CCAGGAGGGTTTAGATATAATCCAGGCGGTCCAGGGTTCGGCTCT CCCGGCATACCCTCCAAAAAAGGAGCTGAATAA
45	Protease D-alanyl- D-alanine carboxypeptidase/ endopeptidase AmpH precursor amino acid (RXF01040; PROKKA_04307) <i>P. fluorescens</i>	MFRRLRGIPLLGCLMGSIGCHSQPPAPPPIQKGDYGAIIRYLQT RIPREMARDNVAGLSIALVNGQELIWARGFGLADKDQGVPTPN TAFRAGGISKLLSATAALQLVEQHHLALDAPIQQTLREFYVRSR FHSDQAEADRAITLRRLLSHQSLPSEHLRDLRSTYAMGQMPMR VSGVWLSSLPGSQVAYSNLGYSLVGAAIERSSGKSFEAQLQSSL LTPLRMNQSSFVGTGAQMGFRAHGYEDGKASTDAQVRDLAAGGL WTSPKDLSTRYVRMLFANGTYKGSQILGSASIDAMFTQQNTGNAL DFDCQIGLGWFLAPCGDEPIGPGVRTYQHSGGGDDFVAQLTLLP DQQLAVIIMANDSNAEDMVVSLTTDSLRLMLQAQTGQPVCAADC QAPSHGLKLRHVPAAVDRKRLAGFYATAWGVFRIRDYHARLTGE LAGYDFELLRDEQGWLRQKKILGFWRKDLGELGRVQLDVIQVQ GRQMLTARSHGQRIAGIERIEPPPLPAAWANTVGTQVQLSSHEP

		DAPLSGISVRQEDGFLVIRGQLHGEPLTDYILLPIDNAHAVLAG NGYGLGDTVSRQVNGLSASGYSFKRTQSPHIPSNF
46	Protease D-alanyl- D-alanine carboxypeptidase/ endopeptidase AmpH precursor example nucleic acid encoding SEQ ID NO: 45	ATGTTTCGCAGGTTGCGCGGTATTCCGCTCTTGGGTTGCCTGAT GGGCAGTATCGGTTGCCACTCGCAACCGCTGCCCCGCCGCCGA TTCAAAAAGGCGATTACGGCGCAATCATCCGCTACTTGCAAACC CGCATTCCTCCGGGAGATGGCTCGGGACAATGTGGCAGGTTTGTCT GATTGCGCTGGTCAATGGCCAGGAGCTGATCTGGGCTCGTGGCT TTGGCCTGGCTGACAAAGACCAGGGGGTGCCGGTTCACGCCCAAT ACCGCGTTTCGCGCCGGTGGCATTTCCAAACCTGCTGAGCGCCAC GGCGGCGCTGCAGCTGGTGGAGCAGCACCACTGGCGCTGGATG CACCGATCCAGCAGACCTGCGTGAGTTCTACGTACGTTACGCG TTTCACAGCGACCAGGCCGAGGCGGATCGAGCGATCACTTTGCG GCGCTTGCTCAGCCATCAATCCGGCTTGCCAGCGAGCACCTGC GCGACCTGCGCAGCACCTACGCCATGGGGCAAATGCCAATGCGC GTGTTCGGGTGTGTGGCTGAGCAGCCTGCCGGGGTCCCAGGTGGC GTACTCCAACCTTGGTTATTCACTGGTGGGCGCGGCCATCGAGC GCAGCAGCGGTAAAAGCTTTGAAGCCCAGTTGCAAAGCAGCCTG CTCACGCCCCCTGCGAATGAACCAGTCCAGCTTCGTAGGCACCGG TGCACAAATGGGCTTTTCGCGCCCATGGTTACGAGGACGGCAAGG CCAGCACCGACGCCCAAGTGCCTGACCTCGCCGCCGGTGGCCTG TGGACCAGCCCCAAAGACCTCAGCCGCTACGTACGCATGCTGTT TGCCAACGGCACCTACAAGGGCAGCCAGATCCTCGGCAGCGCTT CTATCGACGCCATGTTTACCCAGCAAAACACCGGCAACGCCCTG GATTTGACTGCCAGATCGGCCTGGGCTGGTTTCTGGCGCCCTG CGGTGACGAGCCCATCGGCCCCGGTGTGCGCACCTACCAGCACA GCGGTGGCGGCGATGACTTCGTCGCCCAATTGACCCTGCTACCG GATCAGCAGCTGGCGGTGATCATCATGGCCAACGACAGCAACGC CGAAGACATGGTGGTGTCACTGACCACCGACAGCCTGCGCCTGA TGCTCCAGGCACAGACTGGCCAGCCCGTGTGCGCCGATGACTGC CAGGCGCCGAGCCACGGCCTCAAGCTGCGCCATGTGCCGGCGGC GGTGGATCGCAAGCGCCTGGCTGGTTTCTATGCGACCGCCTGGG GCGTGTTCCGCATCAGGGATTACCATGCACGCTTGACCGGCGAA CTGGCCGGCTACGATTTTCGAGCTGTTACGTGATGAACAAGGCTG GCTGCGCGCGCAGAAAAAGATCCTCGGCTTCTGGCGCAAGGACC TGGGCGAGTTGGGCCGCTGCAGTTGGATGTAATCCAGGTACAA

		GGCCGCCAAATGCTCACC GCGCGCAGCCACGGCCAACGCATTGC CATCGGTGAACGCATCGAGCCACCGCCCTTGCTGCCGCTGGG CCAACACGGTCGGCACCTATCAGGTGCTCAGCAGCCATGAACCC GACGCGCCATTGAGTGGCATCAGCGTGCGTCAGGAGGACGGCTT TCTGGTGATTCTGTGGCCAATTGCACGGCGAGCCGCTGACCGACT ACATCCTGCTACCCATCGACAACGCCCATGCGGTACTGGCCGGC AACGGTTACGGCTTGGGCGATACCGTCAGCCGCCAGGTCAACGG GCTGAGCGCTTCGGGTTATTTCCTTCAAACGTACCCAATCACCCC ACATACCCTCGAATTTCTAA
47	Autolytic factor Serralysin precursor amino acid (PROKKA_01103; RXF04500) <i>P. fluorescens</i>	MRVPGPTATNSNAGQVPDPRSGISPEGPTQVYTLNSKKTVFTE QAGKHITRSGFKFHDSNGDGKTTLSYRVSKGFTQQADQARQAL QSWQDVANVTFTTEKRQGADGHIDINEMHGTSGGMASLPNRYMSQ TFANVGTANAGANPPRGHYFREVLVHEIGHTIGLEHPGDYDGS NYGRDAAYAGDTRARSVMSSYYSEKNQPGHDFKSLNPSAPMMDDI SAVQKLYGANTKTRNTDTTYGFNSNTNREAYSLSKANDTPIFCV WDGGGNDTLD FSGYSHHQKINLNAESFSDVGALKGNVSVAKGVT LENAVGGKGGDTLIGNHVANRLKGGAGADRLSGGGGADTFVYDH ASDSTPDNPDVILDFASGADKIDVSAVLKRANVSALKFVDRLTG QPGQAVMSYDEGRNEGGLALDLTGNGKADLLIKSIGQIKAADIL AHGDTTAPNPEPKDKPKQPRPQPEEPKPKPESKPKPEPKPEPKP RPDSCEPKPRPDCEPKPRPDCEPKPRPDCEPKPRPDCEPKPRPD PRPDPREPQPRPDPREPQPRPDPREPQPRPDPREPQPCPDPREP QPRPDCEPQPRPDCEPQPRPDPREPRPRPNPREPQPRPDPRE PQPQPRPDPREPYPRPDPREPRPRPNPREPRPRPNPREPQPRPD PREPRPRPDCEPQPRPDPREPRPRPNPREPQPRPDPREPQPRP DPREPRPRPDPREPQPRPDCEPQPRPEPCEPRPRPNPREPQPR PDPCEPKPTPRTPCEPKAVTRNVRPAYGLSAHSGEYRAMQAPA FDSRHFQGGGLAGEFIRRQKRAE
48	Autolytic factor Serralysin precursor example nucleic acid encoding SEQ ID NO: 47	ATGAGAGTGCCAGGACCAACCGCAACGAATTCTAATGCAGGGCA GGTGCCAGATCCGAGGAGTGGCATCAGCCCGGAGGGCCCTACGC AGGTATATACACTAAACAGCAAAAAAACCGTCTTCACTACGGAA CAGGCCGGGAAACATATACCCCGCAGCGGTTTCAAGTTTCATGA CAGTAACGGTGATGGCAAAACCACGTTGTCCTATCGTGTTTCCA AGGGCTTTACCCACAGCAGGCAGATCAAGCCAGGCAGGCACTG CAATCCTGGCAGGATGTCGCTAACGTCACATTCACCTGAAAAAAG

		GCAGGGGGCTGACGGCCATATAGATATCAATGAGATGCACGGAA CCTCTGGGGGTATGGCCTCACTCCCCAACCGCTATATGAGTCAA ACTTTCGCAAATGTCGGAACAGCGAATGCAGGTGCAAACCCCTCC ACGGGGTCATTATTTTCGCGAAGTTCTAGTTCACGAAATAGGCC ACACCATTTGGGCTGGAACACCCGGGGGACTATGATGGCTCTGGT AACTATGGACGGGACGCAGCGTATGCCGGGGATACTCGAGCGCG TTCTGTGATGAGTTACTATTCGGAAAAAAACCAGCCGGGACATG ATTTCAAATCATTGAACCCCTCTGCGCCGATGATGGATGATATA TCGGCCGTTTCAGAACTCTATGGGGCGAATACTAAAAACGCGTAA TACCGATACGACGTATGGATTTAATTCCAATACAAACCGTGAAG CCTATAGTTTGAAGTCGGCTAACGACACACCCATTTTCTGTGTG TGGGATGGTGGTGGTAATGACACATTGGATTTCTCTGGGTATTC ACACCATCAGAAAAATCAACCTCAATGCCGAGTCCTTTTCGGATG TAGGGGCGTTGAAAGGTAACGTTTCCGTTGCCAAGGGCGTCACG CTGGAATATGCAGTGGGCGGTAAGGGCGACGACACACTTATCGG TAATCATGTTGCCAATCGCCTCAAAGGGGGGGCGGGAGCCGACA GACTGTCTGGGGGGGGCGGCGCAGATACCTTTGTTTACGACCAT GCCAGTGATTCCACCCCGGATAACCGTGATGTCATCCTGGATTT TGCGAGTGCGCAGATAAGATTGATGTATCCGCAGTCCTTAAAA GAGCGAATGTCAGTGCTCTCAAGTTTCGTGATCGCTTAACTGGC CAACCCGGCCAGGCTGTGATGAGTTATGACGAGGGCCGCAACGA GGGGGGGCTGGCCCTGGATCTGACAGGCAACGGCAAGGCTGATC TATTAATAAAAAGCATTGGCCAGATAAAAAGCTGCTGATATCTTG GCGCACGGCGATAACAACCGGCCAAACCCCTGAACCCAAAGATCC CAAGCCGCAGCCGCGTCCTCAACCCGAGGAGCCCAAACCCAAGC CTGAATCCAAACCGAAGGAGCCAAAACCGGAGGAACCAAACCG CGTCCGGAATCGTGTGAACCAAAGCCGCGTCCGGATCCGTTGTGA GCCGAAGCCGCGTCCGGATCCGTGCGAGCCGAAGCCGCGTCCGG ATTCGTGTGAGCCAAAGCCGCGTCCGGATCCGTGCGAGCCGAAG CCGCGTCCAGATCCACGGAACCGCAGCCACGTCCGGACCCGCG CGAGCCGCAGCCGCGTCCAGATCCACGGAACCGCAGCCACGTC CAGACCCACGTGAACCGCAGCCATGTCCGGATCCACGCGAACCG CAGCCGCGTCCGGACCCGTGTGAGCCGAGCCGCGTCCGGACCC GTGTGAGCCACAGCCGCGTCCAGACCCACGTGAACCGAGGCCGC GTCCGAACCCACGTGAACCGCAGCCACGTCCGGACCCACGCGAG
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		CCGCAGCCGCGAGCCGCGTCCGGACCCACGTGAACCGTACCCACG TCCAGACCCACGTGAACCGAGGCCGCGCCCGAACCCACGTGAGC CGAGGCCGCGTCCGAACCCACGTGAACCCACAGCCGCGTCCAGAC CCACGTGAGCCGAGGCCGCGTCCGGACCCGTGTGAGCCACAGCC GCGTCCAGACCCACGTGAGCCGAGGCCGCGTCCGAACCCACGTG AACCACAGCCGCGTCCAGACCCACGTGAACCGCAGCCACGCCCG GACCCGCGTGAGCCGAGGCCGCGTCCGGACCCACGTGAACCGCA GCCACGCCCGGACCCGTGTGAGCCACAGCCGCGTCCGGAACCAT GTGAGCCGAGACCGCGTCCGAACCCACGTGAACCGCAACCCACGT CCGGACCCGTGCGAGCCTAAACCAACCCCTCGCACAGATCCTTG CGAGCCGAAAGCTGTCACTCGAAACGTAAGGCCAGCCTATGGCT TGAGTGCCCATTCAGGCGAGTACCGGGCGATGCAGGCGCCAGCC TTTGATAGTCGTCATTTCCAGGGCGGGCTTGCAGGGGAATTCAT TCGACGTCAGAAGCGCGCTGAATAG
49	Autolytic factor S- type Pyocin amino acid (PROKKA_02110) <i>P. fluorescens</i>	MASTRVRFQFRQDESGELRVYGLHTQPGSGADRVPAQARWNVD KSAMVAVLDGISTITWTPNLGPVVSVPSPYPGTPERLDNMFVHPI AVGQDSAISHYPGRDAENITWQDTIISFPADSGVPPLYLVFAKP AVRPLEVDIYGAFSGRLRNLHVDHIPSQAAIRRHLELYAISFT EKQLKEALNNAASIAIPSYIHQKFSEYGWRNTEKKQTLDDADDL RQAADNNFDAIKPYLLDHGFAETDLEMARTRMHKVNENQGWY
50	Autolytic factor S-type Pyocin example nucleic acid encoding SEQ ID NO: 49	ATGGCCAGCACGCGAGTGCCTTTCCAGTTTCGCCAGGATGAGTC CGGTGAACTGCGTGTTTACGGCCTACATACCCAACTGGCAGTG GCGCCGACCGTGTGCCGGTTGCACAAGCCCGGTGGAATGTGGAC AAAAGCGCGATGGTCGCGGTGCTGGATGGCATCAGCATCACATG GACGCCGAACCTCGGCCCGGTGTGTCAGCGTGCCGAGCCCGTATC CCGGAACACCGGAACGCTTGGATAACATGTTTGTTCATCCGATT GCGGTGGGGCAAGATTCCGGCGATCAGTCACTATCCAGGGCGGGA TGCAGAAAACATCACCTGGCAGGATACGATCATTTTCGTTTCCGG CTGATTCCGGTGTGCCCGCGTTGTATTTGGTGTTTGCCAAGCCG GCGGTCAGGCCGTTGGAAGTTGATATTTACGGTGCGTTCACTGG ACGACTGCGCAACGGGTGTCACGTGGATCACATACCCTCGCAGG CAGCGATAAGACGTCATCTCGAACGCTATGCAATAAGCTTTACA GAGAAGCAGCTCAAGGAAGCGTTAAATAATGCTGCGAGCATTGC TATTCCGTCTTACATCCATCAAAAAGTTTAGTGAGACTTACGGTT GGCGAAACACGGAGAAAAAGCAGACGTTAGATGCCGACGATCTT

		CGTCAGGCGGCAGACAACAATTTTGATGCTATCAAGCCATACCT CCTGGATCACGGCTTCGCTGAGACTGATCTCGAGATGGCACGCA CCCGAATGCATAAGGTCAACGAAAACCAGGGGTGGTACTAG
51	Autolytic factor Linear gramicidin synthase subunit D amino acid (PROKKA_02750; RXF07469) <i>P. fluorescens</i>	MNAEDSLKLARRFIGLPLEKRQLFLQALQKEGVDFSRFPI PAGV EVEDRQALS YAQQRMWFLWQLDPASGAYNLP GAVRLSGVLSLPA LEQAFASLVARHETLRTV FQRQADERLAQVAVEPSVAVEHL DFT ALAFDAREQAVNAAATRQSLLPFDLEHG PLLRVQLLKLAEQEHV LLLLTLHHIVSDGWSMNVLIDEFIRCYDAHERDEAPQLPALPIQY SDYALWQRRWLEAGEQARQLEYWQARLGDEHPVLELPTDHP RPA MPSYQGTRHNFAIEPALAAQLRSCAQKHNVTLFMLLLGAFNVLL HRYTGQGDIRVGVP IANRNRTEVEGLIGFFVNTQVLRTEL SGQT RVAELLQGIKEHALGAQAHQELPFERLVEALKIERSLSHTPLFQ VMYNHQPVVADIASVSTASGLELALVEWQGR TTQFDLTLDTYEK SGTLHAALTYANDLFDTP TIERMARHWTRLLQAMVLDGEQRIGE LPMLDAAEQQRLLHTWNHTAEAYPTERGIHHLIEDQARRSPDAP ALVFGTTTTLTYAQLDARANQLAHALGEQGVGPDV LVGICIERSI EMVVGLLAILKAGGAYVPLDPEYPQERLAYMIEDSGIQLLLSQQ SLLASLPVAGIQVIALDQPALWLDGYSSSESPNVALHALNLAYVI YTSGSTGKPKGAGNSHRALVNRLSWMQQAYGLGANDAVLQKTPF SFDVSVWEFFWPLMSGARLVVAAPGEHREPARLIDTIGRHAIT T LHFVPSMLQAFIHEPGVQACASLTRIVCSGEALPLDAQQ QVFAK LPAAALYNLYGPTEAAIDVTHWTCIDEGVDSVP IGRPIANLGTY VLDAQLNFPVPAGVSGELYLGGLARSYHRRPALTAERFVPSPF VTGERLYRTGDRVRQ RADGVIEYLGRLDHQVKLRGLRIELGEIE ARLMQHPHVREAVVLVHGGKQLVAYLVHPGEAPTDLKAWLLSSL PEYMPVTHFIALPKLPVTANGKLD RKALPVPDAALQQAFVAPQG DLQTALAAIWS DVLGVVEEVGQDDNFFELGGDSIISIQVVSRRARQ AGIRLSPRDLFQYQSIRSLALVARFEQVSLIDQGPVSGEVM LTP VQHSFFDQPIPARHHWNQSLLLVPGEVLEPARLEATLARLIEHH DALRLRFVQQADGWQQSHAAYVSEPLLWQCQASTDAELAALCDE AQRSLDLAQGP LLRAALVN LADGSQRVLLVIHHLVVDGVS WRIL LEDLQQAYRDQALPAKTSAYQRWAQQLHRHAQSLDQQLPYWQAQ SIDAELPCDHPEGGLQNRLGAKLETRLDVEHTRRLQLDAPAA YR TQVNDLLL TALARVISRWSEQPAALI QLEGHGREDLFDDIDL SR TVGWFTSLYPVRLHAEGELSAAIKSVKEQLRAVPNKGIGYGLLR

	YLGTPDTREALSTLAAPRITFNYLQGFDRQFNDLSALFVPARQGS GQAQDAEAPLANWLTVEGQVYGGELSLQWGFSSREMFEAATVQRL ADEYAAELNALIEHCCATPAGQVSPSDFPLARLTQQQLDALPVA GPAIADLYPLSPMQQMLFHTLLEPEAQAYINQLRLDIEGLDVL AFGRAWQAALDRHDI LRSSFHWLGLDSAHLIQRQVDLQLQVIE DPNADFDTLAHAERERGFALNAAPLFRLTIVRGAGAAWHFIFTS HHILMDGWSNAQLLAEVIAHYAGQAVPAPLGQFRDYLAWLQQQS SGEAFWKITALAALPAPTLLAQALRTPVDGVMADHHVALESNFT RRLGEFARQHKVTLNLTLLQGAWSLLLQRYTGQDCVAFGATVAGR SAPLPGIEQQLGLFINTLPIISAASPAQSAATWLSELQVLNLSL RDHEHVPLYDIQGWAGQQGALFDTLVLFENFPVAEALKQGAPAG LTFGRHLNHERTHYPLTLGIELGASLRLEFSYDRAQFSEAQVAQ LSANLQHLLAQLLADAHMPLGNLRLLDAPAQQMLALSRSAAAP QANERVHQRIAAQAEATPDALAVQAGDASVSYAQLNQRANRLAH RLLALGVGPGQVRGLASRRGPQLIVSLLAVLKSGAAYVPLDPEY PAERLAYMLADSRLDLLLSETGLLADLPLPRGLTRVDFSACGEE LTGYPTTNPPNHAAAADLAYVIYTSGSTGQPKGVAIDHAALGQF CDSATLYSRLSAEDRVLQFATFSFDGFVEQCFPPLCAGAALIMR GDELWDAGQLAREIVEQGVTLADLPAAYWYLLAQECAEHRRSLG KLRQVHVGGEAMSVGEVRAWYAAGLGNVRLVNTYGPTEATVVS VHECQLADANDAYGVPIGQAIAGRALYVLDNGFELLATDGVGEL CIGAEVGLAQRYFDRPALTAERFLPDPISATPGARLYRSGDLAR YNPAGALEYVGRIDHQVKIRGLRIEMGEIEASLQALSNVREAAV LAQPSATGVQLVAYVVPAGEQALATQALAARLRQTLPDYMPVGH WVALDALPLNHNKGKLDRRALPTPDLNQASTTYVAPQSPLQIQLA AIWQAVLQVEQVGLEDHFFERGGHSLLATQVISRVRHDLKLEVP LRALFEQPTLAAFAAACAGVQVDTAPVIQAVGRDQPLALSFAQE RQWFLWQLDPTSAAYHVPTALHLRGELDIAALERAVEALVQRHE PLRTTFVESGEHTVQVIHPSLAVPVEQQKVDAGTIEQAVIEEIQ RPFDLRNGPLMRVKLLIVAPDHHVLVITQHHSISDGWSMQVMID EWVALYQGDVGLPALPIQYADYAQWQRDWMAGEQQRQLDYWCA RLGHEHSLLDLPLDHPRAVQSHRGARRQIHLEVRLLTELKALA QRQDVTLEMLLLASFQTLHRYSGQAQVRVGPVANRRNFETER LLGFFVNTQVLQADVHGQMPFDQLLAQVKLRALAEQAQHDLPFE QLVQVLQPERSLSHNPLFQVMFNHQDSLRSAPVQLPGLALQPVD
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		WAGHSTQFDLNLETEESVDGLWASLTATDLFDAATAERLAEHW QNLLRAVLQDASVALDDLAMLSPSQSQQMVHDWNRSDTDYPRER CVHQLFEAQAAAQPDALALHFNDERLSYGELNRRANRLAHLRID MGVGPDLVAVHVERS LDMVVGLLATLKAGGAYVPLDPQFPAER LAYMLEDSRARVLLTQPHLLGHLAQPHGVQVLMVEEAGTAQHNP QVAVTPEHLAYVIYTSGSTGKPKGVMVRHKALCSFTSAMAGTLS IGQDARLLSLTTFSDIFALELYVPLSVGGTVLLSAQAMALDPE AILDLAQRQAANVLQATPSTWRMLLDS PRAHALRGIACLCGGEA LPVDLAQRMLDLQGPLWNLYGPTETTIIWSAAHRLHQALPFVGRP IANTRLFILNAGLTCPQGVSGELLIGGVGLARGYHGPALTAE RFVNPFP GASGERLYRTGDLARYQADGVVEYIGRVDHQVKVRGF RIELGEIEACLREFDGVREAVVLADNDRLIAYLVSTAPQAPQVY KAALRERLPDYMVPAQWLF LDSLPLTPNGKLD RKALPKPDASLS LKGHVAPVTPREQQVA AIWAEVLELPRVGLDDHFFELGGHSLLA TRVVS RVRQALALEVPLKALFEQPLLGD FVRALGEEGVTAPALI KADRTQPLPLSYAQERQWFLWQLDPAGAAHYHIPSALRLQGPLDL TALQESFDSL LARHESLRTYFRQDATGAVQVIDAQSRVDIEQVD SDYAGLKARVAQVVAQPFDLLRGPLLRVTLLRLAEDDHVLVLVQ HHIVSDGWSMQLMVEELVQAYAANSQGQDVQLPTLP IQYADYAV WORDWMEAGEQARQLAYWREQLSGEQPVLELFPDHPRAQPSHR GARLGIELHPELLGSLRALAQHAGVTLPMLLLASYQALLHRYSG QEDVRVGVP IANRNRLTEGLIGFFVNTQVLKADIHGQMSTEQL LHQVRQRSLEAQAHQDLPFQQLVQALQPERSLSLSPLFQVLFNH RVSAADSHLHRLADLHVEVLDLDEGVAQFDLALDVEESPTALRA SLSYATDLFAVATIERMAGHWQNLLRAMVVDPQQPISQLSLLGE DEQQQILELWNQTDAGFSAERLVHELVGDRARETPDAVAVKFDA QTL SYGELDRQANRLAHAL IARGVGREVRVAIAMPRSAESMVAF LAVMKAGGVYVPLDIEYPRDRLLYMMQDSRAQLLLTHSRALQQL PVPEGLETLAIDRTEEWAGYS DTAPDVALDGDNLAYVIYTSGST GLPKGVAVSHGPLVAHIIATGERYETSPADCELHFMSFAFDGSH EGWMHPLINGASVLIRDDSLWLPEYTYEQMHRHNVTMVFPVY LQQLAEHAERDGNPPAVRVYCFGGDAVAQASYDLAWRALKPKYL FNGYGPTETVVTPLLWKARKGDPCGAVYAPIGTLLGNRSGYVLD AQLNLQPIGVAGELYLGGEGVARGYLERPALTAERFVPDPFGKP GSRVYRSGDLTRGRPDGVVDYLG RVDHQVKIRGFRIELGEIAR
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		LREQASVGETV VVVAQEGPTGKQLVAYVVPADASLADPVEFRDAL RRALKADLPDYMVPSHFVFLAQMPLTPNGKLDKGLPLPDASQM QQOYLAPQTELEQQIATIWADILHLPQVGLNDNFFDVGGHSLLA IQITSRVQAEGLDVPLMELFQTESLRAYVQAAATFRAGSVEDEF DDLRDFLSELEAI
52	Autolytic factor Linear gramicidin synthase subunit D example nucleic acid encoding SEQ ID NO: 51	ATGAATGCTGAAGACTCCTTGAAACTTGCTCGCCGTTTATCGG GCTGCCCCCTGGAAAAACGCCAATTGTTCTGCAAGCCTTGCAGA AAGAAGGCGTGGATTTTTCAAGGTTTCCGATTCCGGCAGGGGTG GAGGTGGAGGACCGCCAGGCGCTGTCCTACGCACAGCAGCGCAT GTGGTTTCTATGGCAGTTGGACCCGGCCAGTGGCGCCTACAATT TGCCCGGCGCGGTGCGTTTTAAGTGGCGTGTGAGCCTGCCAGCG CTGGAGCAAGCGTTCGCCAGCCTGGTGGCGCGTCACGAAACCCT GCGCACAGTGTTCCAGCGTCAGGCCGATGAGCGGCTGGCGCAGG TGGCGGTGGAGCCGTCGGTGGCCGTCGAGCACCTGGACTTCACC GCCTTGGCCTTTGATGCGCGGGAGCAGGCCGTCAACGCCGCCGCG CACCCGTCAATCGCTGTTGCCGTTGACCTGGAACATGGGGCCAC TGCTGCGCGTGCAACTGCTCAAGCTTGCCGAGCAGGAACACGTG CTGCTGCTGACCCTGCACCACATCGTCTCCGACGGTTGGTCGAT GAATGTGCTGATCGACGAATTCATCCGTTGCTATGACGCCCACG AGCGCGACGAAGCGCCCCAACTGCCGGCGCTGCCCATCCAATAC AGCGACTACGCCCTGTGGCAGCGCCGCTGGCTGGAAGCGGGCGA GCAGGCGCGCCAATTGGAATATTGGCAGGCCCGCCTGGGTGATG AGCATCCGGTGCTGGAAGTGGCCACTGATCACCCACGCCCCGCG ATGCCCAGCTACCAGGGCACACGGCATAACTTCGCGATTGAGCC GGCAGTGGCCGCGCAACTGCGCAGTTGCGCGCAAAAACACAACG TTACCCTGTTTCATGCTGCTGCTCGGTGCCTTCAATGTGCTGTTG CACCGCTATACCGGCCAGGGCGACATTCGCGTCGGTGTGCCGAT TGCCAATCGCAATCGCACCGAAGTCGAGGGCCTGATCGGTTTCT TCGTCAACACCCAGGTGTTGCGCACCGAACTGAGCGGGCAAACC CGGGTTGCCGAGTTGCTGCAAGGTATCAAGGAGCATGCCCTGGG CGCCCAGGCTCATCAGGAATTGCCCTTTGAACGTCTGGTGGAAG CGCTGAAAATCGAGCGCAGCCTGAGCCACACGCCGCTGTTTCAG GTGATGTATAACCATCAGCCGGTAGTCGCCGACATCGCCTCGGT CAGTACCGCATCGGGTCTGGAATTGGCCCTGGTGGAATGGCAAG GCCGTACCACCCAGTTGACCTGACCCTGGACACCTATGAAAAG

		TCCGGCACCCTGCATGCCGCGCTGACCTACGCCAATGACTTGTT CGATACGCCCACCATCGAGCGCATGGCCCGGCACTGGACCCGCC TGCTGCAAGCTATGGTGCTCGATGGCGAACAGCGCATTGGCGAA TTGCCCATGCTTGACGCGGTGAACAGCAACGGTTGCTCCACAC CTGGAACCACACCGCCGAGGCGTACCCGACCGAGCGCGGCATT ATCACCTGATCGAAGACCAGGCACGGCGCAGCCCCGATGCTCCG GCACTGGTGTTGCGGTACCACCACCTTGACCTACGCCAACTGGA TGCGCGCGCCAACCAATTGGCCCATGCCCTGGGCGAGCAGGGCG TAGGGCCCCGACGTATTGGTGGGTATCTGCATCGAGCGCTCCATC GAAATGGTGGTTGGCCTGCTGGCGATTCTCAAGGCCGGTGGCGC CTACGTGCCCTCGACCTGAGTACCCCCAGGAACGCCTGGCCT ACATGATCGAAGACAGTGGCATTGAGTTGTTACTCAGCCAGCAG AGCCTGCTGGCGTCGCTGCCCGTCGCCGGTATCCAGGTGATTGC CCTGGACCAGCCGGCGCTATGGCTCGACGGATACAGCAGCGAAT CGCCGAACGTGGCCCTGCATGCCCTGAACCTGGCCTATGTGATC TACACCTCGGGCTCCACCGGCAAGCCCAAAGGCGCTGGCAACAG CCATCGCGCGTTGGTCAACCGCTTGAGCTGGATGCAACAGGCGT ATGGCCTGGGTGCCAATGACGCGGTCTTGCAAGAAACCCATT AGCTTTGATGTGTGCGGTGTGGGAGTTCTTCTGGCCGCTGATGAG CGGCGCACGCCTGGTGGTTCGCGGCGCCTGGCGAGCACCGTGAAC CGGCGCGCCTGATTGACACCATTGGCCGGCACGCCATCACCACC TTGCACTTCGTGCCGTGATGTTGCAGGCGTTTATCCATGAGCC GGGCGTACAGGCGTGCGCGAGCCTCACGCGTATCGTCTGCAGCG GCGAAGCCTTGCCCCTGGATGCGCAACAGCAAGTGTTCCGCAAG TTGCCCGCTGCGGCGCTGTACAACCTCTATGGCCCGACCGAGGC GGCCATCGACGTACGCACTGGACCTGCATTGACGAAGGCGTCG ACAGCGTGCCCATCGGCCGCCCCATCGCCAACCTCGGCACCTAC GTGCTGGACGCACAACCTCAACCCGGTGCCGGCTGGCGTCAGCGG CGAACTCTATCTCGGCGGCGTTGGCCTGGCGCGCAGTTACCACC GACGCCCGGCGCTGACCGCCGAACGTTTTGTGCCAGCCCGTT GTGACGGGCGAGCGCCTGTATCGCACCGGTGACCGCGTGCGCCA ACGTGCCGATGGGGTGATCGAATACCTCGGCCGTCTCGATCATC AGGTCAAGTTGCGCGGCTTGCGTATCGAGCTGGGCGAAATCGAA GCACGCCTGATGCAGCATCCACACGTGCGCGAAGCCGTGGTACT GGTACATGGCGGCAAGCAGTTGGTCGCTATCTGGTGACCCAG
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		GCGAGGCGCCAACGGACCTCAAGGCCTGGTTGCTCAGCAGCTTG CCGGAATACATGGTGCCGACGCATTTTCATCGCGCTGCCCCAAGCT GCCGGTGACCGCCAATGGCAAGCTCGATCGCAAGGCGTTGCCAG TGCCAGACGCGGCACTGCAACAGGCGTTTGTGCCCCCAAGGC GACCTGCAAACAGCCCTGGCTGCCATCTGGAGCGACGTACTGGG CGTTGAGGAGGTGCGCCAGGACGATAACTTCTTCGAGCTGGGCG GCGATTCGATCATCTCCATCCAAGTAGTCAGCCGCGCCCGTCAG GCCGGCATTGCGCTGAGCCCGCGTGACCTGTTCCAGTACCAGAG CATCCGCAGCCTGGCCCTGGTGGCGCGCTTTGAGCAGGTCCAGCC TGATCGACCAGGGCCCCGGTCAGCGGCGAGGTGATGCTGACGCCC GTGCAACACAGCTTTTTCGACCAGCCGATCCCGGCGCGGCATCA CTGGAATCAATCCTTGTGCTGGTGCCCGGCGAGGTGCTTGAGC CTGCACGGTTGGAGGCAACGCTGGCGCGGTTGATCGAGCATCAC GACGCCTTGCGCCTGCGTTTTTGTGCAGCAGGCTGACGGCTGGCA GCAGAGCCATGCCGCTACGTCAGCGAACCCTGTTGTGGCAAT GCCAGGCCAGCACCGACGCCGAACCTGGCGGCGCTGTGTGATGAA GCCCAGCGCAGCCTTGACCTTGCCCAAGGCCCGCTGCTGCGCGC CGCGTTGGTGAATTTGGCCGATGGCAGCCAACGTGTGCTGCTGG TGATCCACCACCTGGTGGTGGATGGCGTGTCTGGCGCATCCTG CTTGAAGACCTGCAACAGGCCTACCGCGACCAGGCGCTGCCGGC GAAAACCAGTGCCCTACCAGCGCTGGGCGCAACAGTTGCACCGCC ACGCGCAGTCCCTCGACCAGCAACTGCCGTACTGGCAAGCCCAA TCCATCGACGCCGAGCTGCCGTGTGATCACCCTGAAGCGGCCCT GCAAAACCGCCTGGGTGCCAAGCTGGAAACACGCCTCGACGTCG AGCACACCCGCGACTGCTGCAAGACGCGCCAGCGGCTATCGC ACCCAGGTCAACGACCTGCTGTTGACCGCCCTGGCGCGGGTGAT CAGCCGTTGGAGCGAGCAACCTGCTGCGCTCATTC AATTGGAAG GTCATGGTCGGGAAGACCTGTTTGACGACATCGACCTGAGCCGC ACCGTCGGCTGGTTCACCAGCCTGTACCCGGTGCGCCTGCACGC CGAAGGGGAACGTGTCGGCGGCGATCAAGTCGGTGAAGGAGCAAC TGCGCGCCGTGCCGAACAAAGGCATTGGCTACGGCCTGTTGCGT TACCTCGGCACGCCTGACACCCGCGAAGCGTTGTCGACCCCTGGC CGCGCCGCGCATCACGTTCAACTACCTGGGCCAGTTGACCCGCC AGTTCAATGACTCGGCACTGTTTCGTGCCGGCCCCGCCAGGGCAGT GGGCAGGCTCAGGATGCAGAGGCACCGCTGGCCAACCTGGTTGAC
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		GGTGGAAGGGCAGGTGTATGGCGGTGAGCTGTCGCTTCAATGGG GCTTCAGTCGAGAGATGTTTCGAGGCGGCAACTGTGCAGCGTCTG GCGGATGAGTACGCAGCCGAACTCAATGCGCTGATCGAGCATTG CTGTGCCACGCCGGCAGGCCAGGTGAGCCCGTCGGATTTCCTCGC TGGCACGCCTCACCCAGCAGCAACTGGATGCGTTGCCCGTGGCC GGGCCGGCGATTGCCGACCTTTATCCGCTGTCGCCGATGCAGCA AGGCATGCTGTTCCACACCCTGTTGGAACCCGAGGCCCAGGCCT ACATCAACCAGTTGCGCCTCGACATCGAGGGCCTCGATGTGCTC GCTTTCGGGCGTGCTGGCAGGCTGCACTGGATCGTCATGACAT CCTGCGCAGCAGCTTCCATTGGCTGGGCCTGGACAGTGCTCATC AGCTGATCCAGCGCCAGGTGACCTGCAACTGCAAGTGATCGAA GACCCAAACGCCGACTTCGACACCCTGGCCCACGCCGAACGCGA ACGTGGTTTTTGCCCTGAATGCCGCGCCGCTGTTTCGCCCTGACGC TGGTGCGTGGTGCCGGTGCGGCCTGGCACTTTATCTTTACCAGC CACCACATCCTCATGGACGGCTGGAGTAACGCGCAGTTGCTCGC CGAGGTCATCGCGCATTATGCGGGGCAGGCAGTACCGGCGCCGC TCGGGCAGTTCCGCGATTACCTCGCCTGGCTGCAACAACAGTCC TCGGGCGAGGCGTTCTGGAAAACCGCCCTGGCGGCATTGCCGGC GCCGACTCTGCTGGCGCAAGCGCTGCGCACGCCGGTTCGACGGGG TGGGCATGGCTGACCATCACGTGGCACTGGAGAGCAACTTTACC CGCCGCCTCGGCGAGTTGCGACGCCAGCACAAAAGTCACCCCTCAA TACCCTGTTGCAAGGGGCTTGGAGCCTGTTGCTGCAACGCTACA CCGGCCAGGACTGCGTCGCCTTCGGTGCCACGGTGGCCGGGCGT TCCGCGCCGCTGCCGGGGATCGAGCAGCAACTGGGCCTGTTTCAT CAACACCTTGCCGATCATCAGCGCAGCCTCGCCAGCCCAGTCGG CTGCGACTTGGCTCAGCGAACTGCAAGTGCTCAACCTCAGCCTG CGCGACCATGAACATGTGCCGCTCTACGACATCCAGGGCTGGGC CGGCCAGCAAGGCGCGCTGTTGACACCTTGCTGGTGTTCGAGA ACTTCCCGGTGCGCGAGGCGCTCAAGCAAGGCGCGCCGGCCGGC CTGACCTTCGGTCGCCTGCACAACCATGAGCGCACGCACTATCC ATTAACCTTGGGCATCGAACTGGGCGCCAGCCTGCGCCTGGAGT TCAGCTATGACCGTGCCAGTTTCAGCGAGGCGCAAGTGGCGCAG TTGAGCGCCAACCTGCAACACCTGCTGGCGCAATTGCTCGCAGA CGCTCACATGCCGCTGGGCAACCTGCGCCTTCTCGACGCCCTG CGCAACAGCAGATGCTCGCGCTGAGCCGCTCAGCCGCAGCGCCA
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		CAGGCCAACGAGCGCGTGCATCAGCGTATAGCCGCCCAGGCCGA GGCGACGCCGGACGCCCTGGCTGTGCAGGCCGGTGACGCCAGCG TGAGCTACGCCAGTTGAACCAGCGCGCCAACCGCTGGCCCAT CGCCTGTTGGCGCTGGGTGTCGGCCCCGGCCAACGGGTGGGCCCT GGCTTCGCGGCGTGGCCCGCAGTTGATCGTCAGCCTGCTGGCAG TGCTCAAAAGCGGGGCGGCCTACGTACCGCTGGACCCTGAATAC CCGGCGGAGCGTTTGGCCTACATGCTCGCCGACAGCCGCTGGA CCTGCTGCTCAGCGAAACCGGCTTGCTCGCCGACTTGCCCTTTCG CCCGCGGCTGACCCGCGTGGATTTACAGCGCTGTGGCGAGGAG CTCACCGGCTACCCGACGACCAATCCGCCTAATCACGCAGCGGC GGCTGACCTGGCCTACGTGATCTACACCTCTGGCTCCACCGGCC AGCCCAAGGGTGTGGCCATCGACCATGCCGCCCTCGGCCAGTTC TGCGACAGCGCCACGCTGTACAGCCGACTGAGCGCCGAGGACCG CGTGTTGCAGTTTTCGACCTTCAGTTTCGATGGTTTTGTGGAAC AGTGCTTCCCGCCCCTGTGTGCGGGTGCGGCGTTGATCATGCGT GGCGATGAACTCTGGGACGCCGGGCAACTGGCGCGAGAAAATCGT TGAGCAGGGCGTGACTCTGGCCGATTTGCCCGCCGCCTACTGGT ACCTGTTGGCGCAGGAATGCCCGGAGCACCGTCGCTCCCTGGGC AAGCTGCGCCAGGTGCATGTGGGTGGTGAAGCCATGTCAGTGGA AGGCGTGCGTGCGTGGTACGCCCGGGGCTTGGGCAATGTGCGCC TGGTCAACACCTACGGGCCCACGAAGCCACGGTTGTGTCCAGT GTGCATGAGTGCCAATTGGCCGATGCCAACGACGCCTACGGCGT GCCAATCGGGCAGGCGATTGCCGGGCGCGCGCTGTATGTACTCG ACAACGGTTTCGAACTGTTGGCCACCGATGGCGTGGGCGAGCTG TGCATTGGCGCCGAGGTTGGCTTGGCGCAACGCTACTTCGACCG CCCGGCGCTGACCGCCGAGCGCTTCTTGCCGGACCCGATTTCG CCACGCCCGGTGCGCGGCTTTATCGCAGTGGCGACCTGGCCCCG TACAACCCGGCGGGTGCCTGGAGTACGTGGGGCGTATCGACCA TCAAGTGAAGATTGTTGGCCTGCGTATCGAAATGGGCGAAATCG AAGCCAGCCTGCAAGCCTTGTCCAATGTGCGCGAAGCCGCCGTG CTTGCGCAGCCGAGCGCGACCGGCGTGCAGTTGGTGGCGTACGT GGTGCCAGCCGAAGGCCAAGCGCTGGCGACCCAGGCACTGGCAG CGCGCTTGCGCCAGACATTGCCGGACTACATGGTGCCGGGCCAT TGGGTGGCCCTTGATGCCTTGCCGTTGAACCACAACGGCAAGCT CGACCGCGGTGCACTGCCCCACCCGACCTGAACCAAGCCAGCA
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		CCACCTACGTGGCGCCGCAGAGTCCATTGCAAATCCAGTTGGCG GCGATCTGGCAAGCAGTGTTGCAGGTCGAGCAGGTTGGCCTGGA AGATCACTTCTTCGAACGCGGCGGCCACTCTTTGTTGGCTACCC AAGTGATCTCCCGGGTGCGCCACGACCTCAAGCTGGAAGTGCCG TTGCGGGCGCTGTTTGAACAGCCGACGCTGGCAGCCTTTGCTGC GGCGTGCGCAGGCGTGAGGTCGATACGGCGCCCGTGATCCAGG CAGTTGGCCGTGACCAGCCACTGGCGTTGTCATTTGCTCAGGAA CGGCAGTGGTTTCTCTGGCAATTGGATCCCACCAGCGCGGCCCTA TCATGTGCCCACCGCTTTGCACCTGCGCGGTGAACTCGACATCG CGGCGCTGGAGCGCGCCGTGAGGCCCTGGTGCAGCGCCATGAA CCGCTGCGCACCACTTTGTGGAGTCGGGCGAGCACACTGTGCA AGTCATCCACCCAAGCCTGGCAGTGCCGGTTGAGCAACAAAAGG TCGACGCCGGCACTATCGAGCAGGCTGTCATCGAAGAGATCCAG CGCCCGTTTCGACCTGCGCAACGGCCCCGCTGATGCGCGTCAAGTT GCTGATCGTCGCGCCTGATCACCATGTGCTGGTTATCACCCAGC ACCACATCATCTCTGACGGCTGGTCGATGCAAGTGATGATCGAC GAGTGGGTGGCGCTGTACCAAGGCGACGTTGGCTTGCCGGCCTT GCCGATCCAGTACGCCGACTACGCCAGTGGCAGCGCGACTGGA TGCGGGCTGGGGAACAACAACGCCAGCTCGATTACTGGTGCGCT CGTCTGGGTACGAACATTGCTGCTGGACCTGCCCCCTCGACCA TCCGCGCCCCGCAGTGCGAGCCATCGTGGCGCGCGCCGCCAGA TTCATCTGGAACGTGTGCTGTTGACTGAACTCAAGGCCCTGGCC CAGCGTCAGGACGTGACGCTGTTTCATGCTGTTGCTGGCCTCGTT CCAGACCTTGCTGCACCGCTACAGCGGCCAGGCGCAGGTGCGTG TCGGCGTGCCGGTCGCCAACCGTAATCGCTTCGAAACCGAACGA CTGCTGGGCTTTTTTCGTCAACACCCAGGTGCTGCAAGCTGACGT GCACGGACAGATGCCGTTTCGACCAGTTGCTGGCCAGGTCAAGC TGCGTGCCCTGGAGGCGCAGGCCCATCAGGACCTGCCGTTTCGAG CAGCTCGTGCAAGTGTTGCAGCCCGAGCGCAGCCTGAGCCATAA CCCGCTGTTCCAGGTGATGTTCAACCATCAGGACAGCCTGCGTT CAGCGCCGGTGCAATTGCCCCGGCCTGGCTTTGCAGCCCGTGGAT TGGGCGGGTCACAGCACGCAGTTTCGACCTGAACCTGGAAACCGA GGAATCGGTGGACGGTCTCTGGGCCTCGCTGACTTACGCCACGG ATCTGTTTGACGCGGCGACCGCGGAACGCCTGGCCGAGCATTTGG CAAAACCTGCTGCGCGCGGTCTCTGAGGATGCCTCGGTGGCCTT
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		GGACGACCTGGCGATGCTCAGCCCGTCGCAATCGCAGCAAATGG TGCACGACTGGAACCGCAGCGACACTGACTACCCGCGCGAACGC TGCGTACACCAGTTGTTTGAGGCCCAGGCCGCGGCGCAACCCGA CGCCATTGCGCTGCACTTCAATGACGAGCGCCTGAGCTACGGCG AACTCAACCGCCGCGCCAATCGGCTGGCCCATCGTCTGATCGAC ATGGGCGTCGGCCCGGACGTGCTGGTGGCGGTGCACGTGGAGCG TTCCCTGGACATGGTGGTTGGCTTGCTCGCGACCCCTCAAGGCCG GTGGCGCCTATGTGCCGCTCGACCCACAATTCCCGGCAGAGCGC CTGGCCTACATGCTTGAAGACAGCCGCGCCCGGTATTGCTGAC GCAACCGCACCTGCTGGGGCACCTGGCGCAGCCGCACGGCGTGC AGGTGCTGATGGTGGAGGAGGCCGGCACAGCGCAGCACAATCCC CAGGTTGCCGTGACACCGGAGCATCTGGCCTACGTGATCTACAC CTCTGGCTCCACTGGCAAGCCCCAAAGGGGTGATGGTTTCGTCACA AGGCGCTGTGCAGCTTCACCAGCGCCATGGCCGGCACGTTGAGT ATCGGCCAGGATGCGCGGCTGTTGTGCTGACCACCTTCTCGTT CGACATTTTCGCCCTGGAGCTGTATGTGCCGCTGAGTGTGCGCG GTACCGTGTTGCTGAGCGCCCAGGCAATGGCCCTCGACCCGGAG GCGATCCTCGATCTGGCCCAGCGCCAGGCGGCGAATGTGCTGCA AGCCACGCCCTCGACCTGGCGCATGTTGCTCGACAGCCACGGG CTCATGCACTGCGTGGCATCGCCTGCCTCTGCGGTGGCGAAGCG CTGCCCCTCGATTGTTGGCCCAGCGCATGCTCGATCTGCAAGGCCC GTTGTGGAACCTCTATGGTCCGACGGAAACCACCATCTGGTCGG CGGCTCATCGTTTGCACCAGGCATTGCCGTTCTGTTGGGCGGCCCC ATCGCCAATACCCGCTTGTTTCAATCTCAATGCCGGTCTCACGCC ATGCCCCCAAGGTGTGTCCGGTGAGCTGCTGATCGGCGGTGTGCG GCCTGGCGCGCGGTTACCACGGGCAGCCGGCGCTGACCGCCGAA CGCTTCGTGCCTAACCCGTTTGGGGCATCGGGCGAACGCCTGTA CCGTACCGGCGACCTGGCACGCTATCAGGCGGACGGCGTGGTGG AATACATCGGCCGTGTGACCATCAGGTCAAGGTCCGGGGTTTC CGTATCGAGCTGGGTGAAATCGAAGCCTGCCTGCGTGAGTTCGA CGGCGTACGTGAAGCCGTGGTGCTGGCCGATAACGACCGGCTGA TCGCTTACCTGGTCAGCACCGCGCCGCAGGCACCGCAGGTGTAT AAAGCCGCGCTGCGCGAGCGTCTGCCGGACTACATGGTGGCAGC GCAGTGGCTGTTCTCGACAGCCTGCCGCTGACCCCCAACGGTA AGCTCGACCGCAAGGCACTGCCCAAACCGGATGCCAGCCTGTGCG
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		CTCAAAGGCCATGTAGCGCCCGTCACCCCGCGCGAGCAGCAGGT GGCGGCGATCTGGGCCGAGGTACTGGAATTGCCCCGTGTGGGCC TCGACGATCATTTCTTCGAGTTGGGCGGGCATTTCATTGCTGGCC ACGCGGGTGGTGTACGGGTGCGTCAGGCCCTGGCGCTGGAGGT CCCCTCAAAGCCTTGTTTGAACAGCCGCTACTGGGTGATTTTCG TGCGGGCCTTGGGCGAGGAGGGCGTCACCGCGCTGCGCTGATC AAGGCCGACCGCACGCAACCTCTACCGCTGTCTTATGCCAGGA GCGCCAATGGTTCCCTTTGGCAACTGGACCCGGCCGGCGCCGCGT ATCACATCCCCAGCGCCTTGCGTTTGAGGGGCGCTGGACCTG ACCGCGCTGCAAGAGAGCTTCGATAGCTTGCTGGCTCGCCATGA AAGCCTGCGCACGTATTTCCGTCAGGACGCCACCGCGCGGTTTC AAGTCATTGACGCGCAGAGCCGGGTCGATATCGAGCAGGTTCGAC AGCGACTATGCCGGCCTCAAGGCGCGGGTCGCGCAGGTGGTCGC CCAGCCTTTTCGACCTGCTGCGTGGGCCGCTGCTGCGGGTTACCT TGCTGCGCCTGGCCGAGGACGACCATGTGCTGGTGGTGGTGCAG CATCACATCGTCTCTGACGGCTGGTCGATGCAGTTGATGGTCGA GGAAGTGGTGCAGGCGTATGCCGCTAACAGCCAAGGCCAGGACG TGCAATTGCCGACGCTGCCGATCCAGTACGCCGATTATGCCGTG TGGCAGCGCGATTGGATGGAGGCGGGTGAGCAGGCGCGTCAATT GGCCTACTGGCGTGAGCAATTGAGCGGCGAGCAACCGGTGCTGG AGTTGCCGTTTCGACCAACCGCGCCCGGCACAGCCAAGCCATCGC GGCGCACGCTTGGGTATCGAGTTGCATCCGGAGTTGTTGGGCAG TTTGCGCGCGCTGGCGCAGCACGCTGGCGTCACGCTGCCGATGC TGCTGCTGGCGTCTTACCAGGCATTGCTGCATCGCTACAGCGGC CAGGAAGATGTGCGCGTGGGCGTGCCGATTGCCAACCGTAATCG CCTGGAAACCGAGGGGTTGATCGGCTTCTTCGTCAACACCCAAG TGCTCAAGGCCGATATCCACGGGCAAATGAGCACCGAGCAATTG CTGCACCAGGTGCGTCAGCGTTCCTTCGAGGCCAGGCTCACCA GGACCTGCCGTTTCGAACAGCTTGTCAGGCATTGCAACCGGAGC GCAGCCTGAGCCTGAGCCCGTTGTTCCAAGTGTGTTCAACCAC CGTGTGAGCGCTGCCGACAGCCACCTGCATCGCCTGGCCGACCT GCACGTCGAAGTCCTGGATTTGGACGAGGGCGTGGCCAGTTTCG ACCTGGCGCTGGATGTGGAAGAAAGCCCGACGGCCCTGCGTGCC TCCCTGAGTTATGCCACCGACCTGTTTCGCCGTGGCGACCATCGA GCGCATGGCCGGGCATTGGCAGAACCTGTTGCGGGCAATGGTGG
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		TCGACCCACAGCAGCCCATTAGCCAATTGAGCCTGCTGGGCGAG GATGAGCAACAGCAGATTCTTGAATTGTGGAACCAGACCGACGC CGGTTTTTCAGCCGAGCGCCTGGTGCACGAATTGGTCGGTGATC GCGCCCGGGAAACCCCGGACGCGGTGGCGGTGAAATTCGATGCT CAAACCCTGAGTTACGGCGAGCTGGATCGTCAGGCCAACCGCCT GGCCCATGCCTTGATCGCCCGTGGCGTCGGCAGGGAAGTGCGGG TGGCCATCGCCATGCCGCGCAGTGCCGAGAGCATGGTGGCGTTC CTGGCGGTGATGAAAGCCGGCGGTGTGTATGTGCCGCTGGATAT CGAATACCCACGTGATCGCCTGCTGTACATGATGCAAGACAGCC GTGCGCAACTGCTGCTGACTCACAGCCGGGCGCTGCAGCAACTG CCAGTCCCCGAGGGCCTGGAGACCCTGGCGATTGATCGCACCGA AGAGTGGGCCGGTTACAGCGATACGGCACCGGATGTGGCGCTGG ACGGCGACAACCTTGCCCTACGTGATCTATACCTCCGGCTCCACC GGTTTGCCCAAGGGCGTGGCGGTGTCACACGGGCCGCTGGTGGC GCATATCATCGCTACCGGCGAGCGCTATGAAACCTCACC GGCCG ATTGCGAACTGCACTTCATGTCCTTCGCCTTCGACGGTTCCAC GAAGGCTGGATGCACCCGCTGATCAACGGCGCCAGCGTGTGAT CCGTGACGACAGCCTGTGGCTGCCGGAATACACCTACGAGCAGA TGCACCGCCACAACGTGACCATGGCGGTGTTCCACCGGTGTAC TTGCAACAGTTGGCCGAACATGCCGAGCGCGACGGCAACCCGCC GGCGGTGCGGGTGATTTGCTTCGGCGGTGATGCCGTTGCTCAAG CCAGCTATGACCTGGCCTGGCGCGCGCTGAAACCCAAGTACCTG TTCAACGGCTATGGCCCGACGGAACCGTGGTTACACCGTTGTT GTGGAAGGCCCCGAAAGGCGATCCCTGCGGCGCTGTCTATGCGC CCATCGGCACCTTGCTGGGCAACCGCAGTGGCTACGTGCTGGAT GCGCAACTGAATCTGCAACCCATCGGCGTGGCCGGCGAGTTGTA CCTGGGCGGCGAGGGCGTGGCCCGGGTTACCTGGAGCGTCCGG CACTGACTGCCGAGCGTTTCGTACCGGACCCGTTCCGGCAAACCG GGCAGCCGCGTGATCGCAGCGGCGACCTGACCCGTGGGCGTCC GGATGGCGTGGTGGATTACCTGGGGCGTGTGGACCATCAAGTGA AGATCCGCGGTTTTTCGTATCGAACTGGGGGAAATCGAAGCGCGT CTGCGTGAGCAAGCCAGTGTCTGGTGAAACCGTGGTGGTGGCCCA GGAGGGGCCGACCGGTAAGCAACTGGTGGCCTATGTGGTACCGG CCGACGCCAGCCTGGCCGACCCGTTGAGTTCCGTGACGCCCTG CGTCGTGCCCTGAAAGCCGACCTGCCGACTACATGGTGGCCAG
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		CCACTTCGTATTCTTGGCGCAGATGCCGCTGACCCCCAACGGCA AGCTCGACCGCAAGGGCCTGCCGCTGCCGGATGCGAGCCAGATG CAGCAGCAGTACCTGGCTCCGCAAACCGAGCTTGAGCAGCAGAT CGCCACGATCTGGGCCGACATCCTGCACCTGCCGCAAGTGGGCC TGAACGACAACCTTCTTTGACGTCGGTGGCCACTCCTTGCTGGCG ATCCAGATTACCTCGCGGGTGCAGGCCGAGCTCGGCCTGGACGT ACCGTTGATGGAAGTGTTCAGACCGAATCGCTGCGCGCCTACG TGCAGGCCGCGAGCCACTTTCCGCGCCGGCAGCGTGGAAGATTTT GATGACCTTCGTGACTTTTGGAGCGAAGTAGAGGCGATTTGA
53	Autolytic factor Leukotoxin amino acid (PROKKA_04470) <i>P. fluorescens</i>	MTGGEHFENLVPGTTTPVNTTVTDTPGTDNNTTVTLTAPSAVNEG GQITYTATLSNKAGTDVTLKLDNGSSITIKAGETVGTVPAPT DDVFIDKSTQTVKITETTGGNFEKLEVAGNGATTTINDTIDKVD VVLTAATTVGEGGNIVYTASLVDKNGAPVTNITNPLTVTLDNQG TITIGVNQSSGSITTIAPDDVYKGDQTVTTAIKGVGTGGEHFENL VPGTTTPVNTTVTDTPGTDNNTTVTLTAPAEANEGGQITYTATLS NKAGTDVTLKLDNGSSITIKAGDTVGTVPAPSDDDVFIDKSTQ TVKITDASGGNFEKLEVAGNGATTTINDTIDKVDVVLTAATTVG EGGNIVYTASLVDKNGAPVTNITNPLTVTLDNKGTITIGVNQSS GSVSVLAPDDVYKGDQTVTTAIKGVGTGGEHFENLVPGTTAVNTT VTDTPGTDNNTTVTLTAPSAVNEGGQITYTATLSNKAGTDVTLK LDNGSSITIKAGETVGTVPAPTDDVFIDKSTQTVKITDASGG NFEKLEVAGSGATTTINDTIDKVDVVLTAATTVGEGGNIVYTAS LVDKNGAPVTNITNPLTVTLDNKGTITIGVNQSSGSVSVLAPDD VYKGDQTVTTAIKGVGTGGEHFENLVPGTTTPVNTTVTDTPGTDNT TTVTLTAPSAVNEGGQITYTATLSNKAGTDVTLKLDNGSSITIK AGETVGTVPAPTDDVFIDKSTQTVKITETTGGNFEKLEVAGN GATTTINDTIDKVDVVLTAATTVGEGGNIVYTASLVDKNGAPVT NITNPLTVTLDNKGTITIGVNQSSGSITTVAPNDVYKGDQTVTT AIKGVGTGGEHFENLVPGTTAVNTTVTDTPGSTDLTTVTLTAPTA VNEGGQITYTATLSNKAGSDMLVQLDNNGSSITIKQGETVGTVP PAPTDDVFIDKSTQTVKITGTTGGNFEGVTITPAGATTTINDTI DDVTVVLKATGSVSEGGQIVYTASLVDKNGVAVNNVGSDDLVLK DNGSTITIGNKSTSFTTATAPNDAYVGANDVTTKITGVVSGGD KYEHLIVDGSTVVTKVTDVVSNTTISITGDASVTEGGTAHYTLT LSNPPQTDVTVLKYSATDGSDFNGVYTVKIPAGSSSVPFDI

		RTLDDKITEPTENIVITIDKTTGGNFENLVVGNCSVTTNIIDND APPVIDLDANNSSGASGADFKTTFTTEGGTGVSIADTDIKITDPD STQLTGATVVLTNSQPGDSLNFSGVSGITVTPTTDPVTGKITLT LTGTASLADYMQQIKNITFTNNSHDPSTTPRTITVTVTDGGNYS NVATTTVNVVAVNDAPVATGGAVTGTEDTALALTWANFGVSDVD SPQASLGVKITELPVAGKLQYLAADGSTWTNVTSGQTFTKAQID GGQLRFTPNANESGADGYGGTGVGNKQADYAQFKFQPTDGKDLG TSATVKVDITPVADAPTLVADNNVASTGLVKQGWNSIAGLGNN GNGAAPDVLKKAIDNAGTPNNTSVVTNVESVDNVAAGSGSKISG LIYMEAGKSYTFSGIADDSVVVNVGKDVASGLWGTNSGKFSGS FTPTTTGYYSLEIYQANQAGPGSFDVNLSINGGAVQNLSTSTVP LYTGLTDLTNAGVTVSDLHGSNGDGYVGYKLNQENGTVKLS KVTTALTDTDGSETLSVKISGIPAGSVLTDASGHTFTAGKT'VGE VNVITGWDNLTLTIKPTTYSGQFNLTVTSTSTESIGGSATTTAQ LPVTVHPATYNSVTGTSGSDTINGSDGNDIVVADIAGLNVVQ GK NYNIAFMVDSSGSMVASLDAAKASLTSVFNSLKDSL GANTS GT VNI FLADFDSQVKKSVAINLNDPNALTQLKAVLDSMASGGGTNY EDVFKATANFFQSDLATKNTGATNLTYFITDGKPTYHQSGEQIN PVVTDIFYDFRTTDGRLDDYISANNYVLGNTFSINVNGANLQLID SQGQLHQWKQTFLLGGWYDNGVIGTVHAQGDGTFEVSYLDGSGSS TTTATINNANS GFALLKGLSAVEAIGINGDISLDDLKPYD TDGK PQTNI DPKDLANAILGHTEATLPGADTVSGGDGNDILFGDLVSF SGINGEGYNALQAFVAQKTGVAVSAVTASN VHQYVTEHYVDFDV SGA KDAGDTLLGGAGDDILFGQGGNDTLDGGKGNDILLGGTGND TLIGGQGNDILIGGSGADTFVWKS GDIGNDVIKDFKASEGDRID LRDLLKGETDSTIDNYLKITTVDGVSTLQVSSEGLKNAAGGLAN ADVTIKLEGNDWSHTSINSLISGADPTIKIDHT
54	Autolytic factor Leukotoxin example nucleic acid encoding SEQ ID NO: 53	ATGACCGGCGGCGAGCACTTTGAAAAATCTGGTTCCAGGTACTAC CCCGGTTAACACCACCGTTACGGACACACCGGGTACCGATAACA CCACCACCGTTACGCTGACAGCGCCAAGTGCCGTTAACGAAGGT GGGCAGATTACGTACACCGCAACGCTTTCCAATAAAGCGGGCAC TGATGTCACGCTGAAGTTAGATAACGGTTCGTCGATCACCATCA AGGCCGGCGAAACCGTCGGCACCGTGACTGTCCCTGCGCCTACC GATGACGTGTTTATCGATAAGAGCACCCAGACCGTCAAGATCAC CGAAACCACTGGCGGCAACTTCGAAAAACTCGAAGTGGCAGGAA

		ACGGCGCAACCACCACGATCAACGACACCATCGACAAAGTCGAT GTGGTCCTGACCGCCACTACCACCGTCGGCGAAGGCGGCAATAT CGTCTACACCGCCAGCCTTGTGGATAAGAACGGCGCACCGGTGA CCAACATCACCAATCCGCTGACCGTGACATTGGATAACGGCCAG ACCATCACTATTGGCGTAAACCAGTCGAGCGGTTCTATCACCAC CATCGCGCCAGACGATGTCTACAAAGGCGACCAGACCGTCACTA CCGCCATCAAAGGCGTGACCGGCGGCGAGCACTTTGAAAATCTG GTTCCAGGTACTACCCCGGTTAATACCACCGTTACGGATACACC AGGCACTGACAACACCACTACGGTGACGCTGACCGCTCCGGCCG AGGCAAACGAAGGTGGGCAGATCACGTACACCGCCACGCTTTCC AACAAAGCGGGCACTGACGTAACGCTGAAACTCGACAACGGTTC TTCGATCACCATCAAGGCTGGCGACACTGTTGGCACTGTGACTG TGCCCGCTCCAAGCGATGACGTGTTTCATCGATAAAAGTACCCAG ACCGTCAAGATTACTGACGCTTCCGGCGGCAACTTCGAAAACT CGAAGTCGCAGGTAACGGCGCAACGACCACGATCAACGACACCA TCGACAAGGTGGATGTAGTTCTAACTGCTACCACTACCGTCGGC GAAGGCGGCAACATCGTCTACACCGCCAGCCTTGTGGATAAGAA CGGCGCGCCGGTGACCAACATCACCAACCGCTGACCGTGACAT TGGATAACGGTAAAACCATCACCATCGGCGTAAACCAATCGAGC GGTAGCGTTTCGGTTCTTGCTCCTGATGATGTGTACAAGGGCGA CCAACTGTCAACACCGCTATAAAGGGTGTGACCGGCGGCGAGC ATTTGAGAACCTGGTGCCTGGCACTACGGCAGTGAACACCACT GTCACCGATACCCCCGGTACCGACAACACCACCACTGTGACGCT GACAGCGCCAAGTGCCGTTAACGAAGGTGGTCAGATCACTTACA CCGCGACCTTGAGCAACAAGCCGGTACTGACGTACCTTGAAG CTGGATAACGGCTCTTCGATCACCATCAAAGCTGGCGAGACCGT CGGTACTGTGACCGTGCCCTGCGCCTACCGATGACGTGTTTCATCG ATAAGAGCACTCAGACCGTCAAGATCACCGACGTTTCGGGCGGT AACTTCGAAAACTGGAAGTTGCAGGCAGCGGCGGACCACTAC GATCAACGACACTATCGACAAGGTCGATGTGGTCCTGACCGCCA CCACCACCGTCGGCGAAGGCGGCAACATCGTTTACACCGCCAGC CTCGTGGATAAAAAACGGCGCACCGGTGACCAACATCACCAATCC GCTGACCGTGACCTGGATAACGGCAAGACCATCACCATCGGCG TAAACCAATCGAGCGGTAGCGTTTCGGTTCTTGCTCCGGATGAT GTGTACAAGGGCGACCAAACCTGTCACCACCGCTATCAAGGTGT
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		GACCGGCGGCGAGCACTTTGAAAATCTGGTTCCAGGTACTACCC CGGTTAACACCACCGTTACGGACACACCGGGTACCGATAACACC ACCACCGTTACGCTGACAGCGCCAAGTGCCGTTAACGAAGGTGG GCAGATTACGTACACCGCAACGCTTTCCAATAAAGCGGGCACTG ATGTCACGCTGAAGTTAGATAACGGTTCGTGATCACCATCAAG GCCGGCGAAACCGTCGGCACCGTGACTGTCCCTGCGCCTACCGA TGACGTGTTTATCGATAAGAGCACCCAGACCGTCAAGATCACCG AAACCACTGGCGGCAACTTCGAAAAACTCGAAGTGGCAGGAAAC GGCGCAACCACCACGATCAACGACACCATCGACAAAAGTCGATGT GGTCCTGACCGCCACTACCACCGTCGGCGAAGGCGGCAACATCG TCTACACCGCCAGCCTTGTGGATAAGAACGGCGCACCGGTGACC AACATCACCAATCCGCTGACCGTGACCCTGGATAACGGCAAGAC CATCACCATCGGTGTGAATCAGTCGAGCGGTTCCATCACCAACG TAGCGCCAAACGACGTCTACAAAGGCGACCAAACCGTCACCACC GCCATCAAAGGCGTGACCGGCGGCGAGCACTTCGAGAACCTGGT GCCGGGCACGACGGCGGTGAACACCACCGTCACCGACACACCAG GCTCCACCGACCTGACCACCGTTACCCTGACTGCCCCGACCGCG GTCAACGAAGGCGGCCAGATCACCTACACCGCCACCTTGAGCAA CAAGGCCGGTAGCGACATGCTGGTCCAGCTCGACAACGGTTCGA GCATCACTATCAAGCAAGGTGAGACCGTGGGCACGGTGACCGTC CCGGCGCCTACCGATGACGTGTTTCATCGACAAGAGCACCCAGAC CGTCAAGATCACCGGCACCACCGGCGGCAATTTGAGGGCGTGGA CCATCACACCTGCGGGCGCCACGACCACCATCAACGACACCATC GATGACGTGACCGTGGTACTCAAGGCCACTGGCTCGGTGAGCGA AGGCGGGCAGATCGTGACACCGCGTCCCTGGTCGACAAGAACG GTGTGGCGGTGAACAACGTTGGCTCAGACCTGGTCGTCAAGCTG GATAACGGCTCGACCATTACCATCGGCAATGGCAAGTCCACCAG CTTACCACCGCCACCGCACCTAACGATGCGTATGTCGGCGCCA ATGACGTCACCACTAAAATCACGGGTGTGGTCAGCGGTGGCGAC AAGTACGAACACTTGATCGTCGACGGCAGCACCGTGTTACCAA AGTGACCGATGTGGTCAGCAACACCACCATCAGCATTACCGGCG ATGCGTCGGTGACTGAAGGCGGTACGGCGCACTACACGCTGACC CTGAGCAACCCGCCGCAAACCGACGTGACCGTGACGCTCAAGTA CAGCGGCACCGCTACCGACGGTTCAGACTTCAATGGCGTGTACA CCGTGAAGATTCCGGCAGGCTCCAGCAGCGTACCGTTTGATATC
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		CGCACGCTCGACGACAAGATCACCGAGCCGACGGAAAAATATCGT CATCACCATCGACAAGACCACTGGCGGCAACTTCGAAAACCTGG TGGTCGGCAATGGCAGTGTTACCACCAACATCATCGACAATGAT GCGCCGCGGTCATCGATCTGGATGCCAACAACTCCAGCGGCGC CAGCGGTGCGGACTTCAAGACCACCTTCACCGAAGGCGGCACCG GTGTGTCAATTGCTGACACTGACATTAAGATCACCGACCCGGAC AGCACCCAACTGACCGGCGCCACCGTGGTATTGACCAACAGCCA GCCAGGCGACTCGCTGAACITTCAGCGGCGTGAGCGGCATCACCG TGACCCCGACTACCGACCCCTGTGACCGGTAAAAATCACCTTGACC CTGACCGGGACGGCGTCGCTGGCCGACTACATGCAGCAGATCAA GAACATCACGTTACCAACAACAGCCACGACCCGAGCACCACGC CGCGCACCATCACCGTGACGGTGACCGATGGCGGCAACTACTCC AACGTGGCTACCACCACCGTCAACGTGGTAGCAGTCAACGATGC ACCAGTGGCCACTGGCGGTGCCGTGACCGGTACGGAAGACACCG CGCTGGCCCTGACCTGGGCCAACTTCGGCGTGAGCGATGTGGAC TCGCCACAAGCCAGCCTCGGGGTGAAAAATCACCGAGCTGCCGGT AGCCGGCAAGCTGCAATACCTGGCGGCGGACGGCAGCACCTGGA CCAACGTGACCAGCGGCCAGACCTTTACCAAGGCTCAGATCGAT GGCGGCCAACTGCGCTTTACGCCGAACGCCAACGAGTCCGGCGC CGACGGTTATGGCGGCACTGGCGTGGGTAACAAGCAGGCGGATT ACGCGCAGTTCAAGTTCCAACCAACCGATGGCAAGGACCTGGGT ACCAGCGCCACGGTGAAAGTCGATATCACGCCGGTAGCCGACGC GCCGACCCTGAGCGTGGCAGACAACAACGTTGCCCTCCACCGGCC TGGTCAAACAGGGCTGGAACAGCATTGCCGGCCTCGGCAACAAC GGCAACGGCGCTGCACCGGACGTGCTGAAAAAAGCCATCGATAA CGCGGGCACGCCGAACAACACCTCGGTGGTGACCAACGTCGAGT CTGTCTGACAATGTCGCCCGCGGCTCTGGCTCAAAAAATCTCCGGC CTGATCTACATGGAAGCCGGCAAGAGCTACACCTTCAGCGGCAT CGCCGATGACAGCGTGGTGGTCAACGTTGGCGGTAAAGATGTTG CCAGCGGTTTGTGGGGCACCAACAGCGGCAAGTTCAGCGGCTCG TTCACGCCAACGACCACCGGTTACTACAGCCTTGAGATCTACCA GGCCAACCAGGCGGGCCCAGGCAGCTTCGACGTTAACCTGTCTGA TCAACGGCGGGGCGGTGCAGAACCTGAGCACCAGCACCGTGCCG TTGTACACCGGCCTTACCGACCTGACCAACGCCGGCGTCAACGT ATCCGACCTGCATGGCAGCAACGGTGACGGCTACTACGTGGGCT
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		ACAAGCTCAACGAAGGCCAGGAAAACGGCACGGTCAAACCTGTCC AAGGTCACCACCGCGCTGACCGATACCGACGGCTCCGAAACCCCT GAGCGTAAAGATCAGCGGCATTCCGGCAGGCTCGGTGCTTACCG ACGCGTCGGGGCACACCTTTACTGCGGGTAAAACCGTGGGCGAA GTGAATGTCACCGGCTGGGACCTGAACACCCTGACCATCAAGCC GCCGACCTACTACAGCGGCCAGTTCAACCTGACGGTCACCTCGA CTTCCACCGAGAGCATCGGCGGTTTACGCGACCACCACCGCGCAA TTGCCAGTCACGGTGCATCCGGCGACCTACAATTCTGGTACCCGG CACCTCGGGCAGCGACACCATCAATGGCAGCGATGGCAACGACA TCGTCTGTGGCCGACATCGCCGGCCTGAACGTGGTGCAGGGTAAG AACTACAACATCGCGTTTATGGTGGACAGCTCCGGCAGTATGAG CGTCGCCTCGCTCGACGCGGCGAAGGCCTCGTTGACTTCGGTGT TCAACTCGCTCAAGGACAGCCTGGGCGCCAACACATCGGGGACC GTGAATATCTTCTTGGCGGACTTTGATAGCCAAGTGAAAAAGTC GGTGGCTATCAACCTCAACGATCCTAATGCATTGACTCAGCTGA AAGCGGTGCTGGACTCGATGGCATCGGGAGGAGGTACTAACTAC GAAGACGTGTTCAAGGCCACTGCCAACTTCTTCCAGAGCGACCT GGCGACCAAAAACACCGGTGCAACCAACTTGACGTACTTTCATCA CCGACGGCAAGCCGACCTACCACCAGAGCGGCGAGCAGATCAAC CCGGTAGTGACTGACTTCTACGACTTCCGCACCACCGATGGGCG CTTGGACGACTACATCAGTGCGAACAACATATGTGCTGGGTAACA CGTTCAGCATCAACGTCAATGGCGCTAACCTGCAGTTGATCGAC AGCCAGGGCCAACTGCACCAATGGAAGCAGACGTTTCTGGGTGG CTGGTACGACAACGGCGTCATAGGTACCGTGACGCCCAGGGTG ACGGGACTTTTGAAGTCTCTTACCTCGACGGCTCCGGTAGTAGC ACCACCACCGCGACCATTAAACAACGCCAACAGCGGTTTTGCACT GCTCAAAGGTTTGTCTGGCGGTGGAAGCAATCGGCATCAACGGCG ACATCAGTCTCGACGATCTCAAGCCGTACGATACCGATGGCAAG CCGCAAACCAACATCGATCCGAAGGACCTGGCCAACGCTATCCT CGGCCACACCGAGGCGACGTTGCCGGGCGCGGACACCGTCAGCG GTGGCGACGGCAACGACATCCTGTTCCGGCGACCTGGTGAGTTTC AGCGGGATCAATGGCGAGGGTTACAACGCACTGCAGGCCTTTGT CGCACAGAAGACCGCGTGGCTGTCTCGGCAGTGACTGCCTCTA ACGTTACACAGTACGTCACCGAGCACTATGTGGACTTCGACGTC TCCGGCGCCAAAGATGCCGGCGACACACTGTTGGGCGGCGCTGG
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		CGATGACATCTTGTTCGGCCAAGGCGGCAACGACACGCTCGATG GCGGCAAAGGCAATGACATCCTGCTGGGTGGCACGGGTAACGAC ACGTTGATTGGCGGCCAGGGCAACGACATCCTGATCGGTGGCTC GGGTGCCGACACCTTTGTGTGGAAGTCTGGCGACATCGGCAACG ATGTGATCAAGGACTTCAAGGCGTCCGAAGGCGACCGCATTGAC CTGCGTGATTTGTTGAAAGGTGAAACCGACAGCACCATCGACAA CTACCTCAAGATCACCACGGTAGACGGCGTGTCGACCCTGCAAG TGAGCAGTGAAGGCAAGCTCAACGCCGCCGGTGGCTTGGCCAAT GCCGATGTGACGATCAAGCTGGAAGGCAACGACTGGTCCCACAC CAGCATCAACTCGCTGATCAGTGGTGCCGACCCGACCATCAAGA TCGACCACACTTAA
55	Autolytic factor Hemolysin transporter protein ShlB precursor amino acid (PROKKA_00510; RXF07570) <i>P. fluorescens</i>	MSLFLPRTWLLLGVCLLTGFALNSASAAPT PGDQDLIRDRQNR LEEQQRRLEELKDLPGNEAKPVAPAAPVNTRCFFIKDIELKGAD SLPAADRERLLKPYIGQCLGVSQNLNELLKAITDYIDKGLVTSR AYLPQQDL SKGHLQVLVVEGKLEGLKGADNSKLS DRELAMAFPG KNGDLLNLREIEQAIDQLNRLPSNQAQME LTPGDAVGGSSVLVK NNPQKPWRASLSRNNDGQKSTGEQQWGTGFEWDSPLGLADQLIL RGGHDAISDHQKTSKNVLLYYNVPWGWWNFSYSYNQSDYRSVAQ ADTYNFKQSGDSQNHQLRAERVIHRDAVSKTSVNVGLSHLR TNN YIEDSRLDVSSNRLSELQLGINHGRRIGSAFVNIDLG VQNGIGA FDAQRNDQQRDQRGNLTPDPYRKYTATVSYLQPF TLWGESFSF TSLATGQRSEDVLFSPQRM SLGGSSSIRGFKDQQLTGDSGGYWR NDLRWARPVITWDMRPVFAEY GASVGYDQGVIRNDRYNGEVHGR VSSNSLELFARGKYVSTSVTFAHSLERPAVLTEREAPIYFRMGF FL
56	Autolytic factor Hemolysin transporter protein ShlB precursor example nucleic acid encoding SEQ ID NO: 55	ATGTCTTTATTCTGCCACGGACTTGGCTGCTACTTGGCGTCTG CCTGCTGACTGGCTTCGCGCTGAACAGCGCGTCCGGCTGCACCTA CGCCCGCGATCAGGACTTGATCCGCGACCGGCAAAATCGCCTG CTGGAAGAACAACAGCGGCGTCTTGAAGAGCTCAAGGATTTGCC CGGCAACGAGGCCAAGCCGTCGCTCCCGCGCTCCAGTGAACA CCCGTTGCTTCCCCATCAAAGACATCGAGCTCAAAGGCGCCGAC AGCCTGCCTGCCGCTGACCGCGAGCGCTTGCTCAAGCCCTATAT CGGCCAGTGCTGGGTGTGTCCAGCTCAATGAACTGCTCAAGG CCATCACCGATTACTACATCGACAAAGGCTGGTCAACAGCCGA GCTTACTTGCCGCAACAGGACCTGTCCAAGGGGCACCTGCAAGT

		GTTGGTGGTGGGAAGGCAAACCTCGAAGGTTTGAAAGGCGCCGACA ACAGCAAGCTCTCGGACCGCGAATTGGCCATGGCCTTTCCCGGG AAAAACGGCGACTTGCTGAACCTGCGAGAAATCGAGCAAGCCAT CGACCAACTCAACCGCTTGCCATCCAACCAGGCGCAAATGGAGC TGACGCCAGGTGATGCCGTTGGCGGCAGTTCGGTGCTGGTGAAA AACAACCCACAGAAGCCTTGGCGCGCCAGCTTGTCGCGCAATAA CGACGGCCAGAAAAGCACCGGCGAACAGCAATGGGGTACCGGGT TTGAATGGGACAGCCCATTGGGCCTGGCCGATCAACTGATTCTG CGCGGCGGCCACGACGCCATCAGTGACCACCAGAAAACCTCGAA AAACGTGTTGCTTTACTACAACGTGCCCTGGGGCTGGTGGAAC TCAGCTACAGCTACAACCAGAGCGATTACCGCTCGGTTGCTCAG GCCGACACCTACAACCTCAAGCAAAGCGGCGACAGCCAGAACCA CCAACTGCGCGCCGAACGTGTGATCCACCGCGACGCTGTAAGTA AGACCTCGGTTAACGTGCGCCTATCCCACCTGCGCACCAACAAC TACATCGAAGACAGCCGTCTGGACGTCAGCAGCAATCGCTTGAG CGAACTGCAACTGGGCATCAACCACGGGCGACGGATCGGCAGTG CCTTCGTCAACATCGACCTCGGTGTGCAGAACGGCATAGGTGCC TTCGATGCCCAGCGCAACGATCAGCAGCGCGACCAGCGTGGCAA CCTCACCCCCACCCCGGACTACCGCAAATACACCGCGACCGTCA GCTATTTGCAGCCGTTACGTTGTGGGGCGAGTCCTTCAGCTTT ACCAGCCTGGCCACCGGGCAGCGCAGTGAAGACGTGCTGTTTCAG CCCTCAGCGCATGAGCCTGGGTGGTTCGTCTGTCGATACGCGGTT TCAAGGACCAGCAACTGACCGGCGACAGCGGCGGCTACTGGCGC AACGACCTGCGCTGGGCGCGCCCGGTGACCTGGGATTGGATGCG TCCGGTTTTTTGCCGAATACGGTGCCAGTGTCGGTTACGACCAGG GTGTGATTTCGAATGACCGCTACAACGGGGAAGTGACCGGTCGG GTGTGAGCAACTCGCTGGAGCTATTTGCCCGCGGCAAATACGT CAGCACCAGCGTGACCTTTGCCCATTCCTTGAACGACCGGCGAG TGCTGACCGAGCGCGAAGCGCCGATCTACTTCCGCATGGGTTTC TTCCTGTAA
57	DegP2 example nucleic acid encoding SEQ ID NO: 31	ATGTCGATAACCACGTTTGAAGTCTTACTTATCCATAGTCGCCAC AGTGCTGGTGCTGGGTGAGCCCTTACCTGCGCAAGCGGTGAGT TGCCTGACTTCACCCAACCTGGTGGAGCAGGCCCTGCCTGCCGTG GTGAACATCAGTACCACGCAGAAAGCTGCCGGATCGCAAAGTCTC GAACCAGCAGATGCCCCGACCTGGAAGGCTTGCCGCCCATGCTGC

		GCGAGTTCTTCGAACGAGGGATGCCGCAACCACGCTCCCCCGT GGCGGCGGTGGCCAGCGCGAAGCCCAATCCCTGGGCTCCGGCTT CATCATTTTCGCCTGACGGCTATATCCTCACCAACAACCACGTGA TTGCCGATGCCGACGAGATTCTCGTGCGCCTGGCCGACCGCAGT GAACTCAAGGCCAAGCTGATTGGCACCGATCCACGTTCCGACGT GGCCTTGCTTAAATCGAGGGCAAGGACTTGCCGGTGCTTAAGC TGGGCAAGTCCCAGGACCTGAAGGCCGGTCAGTGGGTGGTCGCG ATCGGTTTCGCCGTTTCGGCTTTGACCACACCGTTACCCAAGGCAT CGTCAGCGCCATCGGTTCGAGCCTGCCGAACGAAAACCTACGTAC CGTTCATCCAGACCGACGTGCCGATCAACCCGGGTAACTCCGGT GGCCCGCTGTTCAACCTGGCCGGCGAAGTGGTGGGGATCAACTC GCAGATCTACACCCGCTCCGGCGGCTTCATGGGCGTGTCTTTCG CGATCCCAATCGATGTGGCCATGGACGTCTCCAATCAGCTCAAA AGCGGCGGCAAGGTCAGCCGCGGCTGGTTGGGCGTGGTAATCCA GGAAGTGAACAAGGACCTGGCTGAGTCCTTCGGTCTCGACAAGC CGGCCGGTGCCCTGGTTGCGCAGATTCAGGACAATGGCCCTGCG GCCAAAGGCGGCCTGAAAGTCGGTGACGTCATCCTGAGCATGAA CGGCCAGCCGATCATCATGTGCGCAGACTTGCCCTCATTGGTTCG GCGCGCTCAAGGCCGCGCGCAAAGCCAAGCTGGAAGTGATTTCGT GATGGCAAGCGCCAGAACGTCGAACTGACCGTAGGTGCCATCCC GGAAGAAGGCGCGACCCCTGGATGCCCTGGGCAACGCCAAGCCCG GTGCCGAGCGCAGCAGTAACCGCCTGGGTATCGCCGTGGTTGAA CTGACCGCCGAGCAGAAGAAAACCTTCGACCTGCAAAGCGGTGT GGTGATCAAGGAAGTTCAGGACGGCCAGCCGCTTGATCGGCC TGCAACCGGGTGACGTGATCACTCACTTGAACAACCAGGCAATC GATACCACCAAGGAATTCGCCGACATCGCCAAGGCGTTGCCGAA GAATCGCTCGGTGTGATGCGCGTCCTGCGTCAAGGCCGTGCCA GCTTCATTACCTTCAAGCTGGCTGAGTAA
58	ShyA <i>Vibrio cholera</i>	MISKSIIILRFSELSMRKKVTLVGLPLLAVAAISSSLNSPTRQQR IELSLPESPLVQFSSAEHTVEVVKVGHDPYEYEIKPGDNLSTIF NQLGFAYTELMKVMETDLNYLALDTLRPGNVLRFWKGSNTLAK MELEFSLVDRAVYTRLNDGSYEFEEERKIPGTWKVEPLIGEVDGS FSLSANRAGLGAADVQIVTLLKDKINFGRDLRRGDRFEVVLRSR QLVGEKLTGNSEIQAIKIFN

		RGKEITAYLHQDGQYYDKNGDSLQRAFRQRYPVDSKWRISSNFDP RRLHPVTKRVAPHNGTDFAMPIGTPVYTSGDGVVVMTRNHPYAG NYVVIQHGNTYMTRYLHLSKILVKKGQKVSQRIGLSGNTGRV TGPHLHYELIVRGRPVNAMKANIPMASSVPKKEMAQFIAKRKEL DQMLARQESMLAAQ
59	Shine Dalgarno ribosome binding sequence	AGGAGG
60	DsbC amino acid (RXF03307; PROKKA_03627) <i>P. fluorescens</i>	MRLTQIIAAAAIALVSTFALADDAAEQTIKRS LANLALDTPIES ISASPMAGLYEVKLKGSRVLYASADGQYIVQGYLFQLKDGKPVN LTEKAERLGVSKLINGIPVAETVVYPAIGETKTHITVFTDTC YCHKLHAEIPALNKLGVVRYVAFPRQGLGSPGDEQLQAVWCSA DKKAAMDKMVDGKEIKSAKCANPVSKQFALGQSIGVNGTPAIVL ADGQVIPGYQPAPQVAKLALGAK
61	RXF04495.2 KO homology arm	GCTTGACGCTGCTGGGCACCGGTGATGCGCGGCAGGTTCCGGTG CATGGCTGCGAGTGTGCTGCGTGCGGGTTGGCGCGCAGTGATCA AAGCCGCCCCAGCCGACGGTGCCGCGTAATCACAATGACCTGAC GCTGGCGCTGCAGAGCATCGAAGACACCGGGGCGCAGTTGGGGG GGGCTGACCCATGTGGGGCATACTGTTGGATACGTGGTTGCTGGC GCATCGTCATGAGTTGCCCCGACATGTCTCGGTAGGTTGGGACA ATCGAGTCGTGTAAGGCGTGGCCTTGTTAGCAATCAGACAAGAA GCTTGATGTTTCAGTTTGTGTTTTTCCAGTGTGTTTGATTGTTTTT CTGGATGTTTGAAGCGTGTGCTTGATTGAGTCAAGTTTGTGTT TTGCACTTTTTTTTCTTCGGTGGCATCAAGTTTGAGAGTGCTT GGGGGATGCGAGTATTCCACCTCGAATAAAACATGTGTGGTTTTT ATTACTGCCATGTTTAATGGTGGGTTGTTGAAATGAAATGTGAG CCCAGTCACTATTGCTAACCCCCCGACAAGCCTGCCAGGC AGGCGTCTGTGTGCCAGGCAACGACCTCCCGTGGGGTTCTCAGT CCAGGGAACCCACGATTGCACTAGAACCTTTCTCTTACTTCTG ACCGTATACGCGTGCGGCGCTGCGTGCTTATCAAGTGAGC ATGGCTACTTTCAAGCCACGTTTCATGTCGTGTTTTTTTACCAA ACTATCAGGGGTTGGTGATGCCTTCCGGTTTTTTTCAAGTTATTCA AAACTCCCGTTGACTCACTCACTGGGTTTATTGCCTGTGCGTTA TTCATGTTCCCGTTTTCAGAGGTGTGCGACTGATCGCCTGTGCA GTGCATTGAATGACTCATGTGCGGCAGACGGAAGTCGCTGTATG

		TGGAATGCTGATTTTTTTCCTTCATGTTCTATTCTATTGTTTCGCC ATTCAAGTTGGTAGTCGCCTGGGGGACGTGAAAAATATGAGGGT GGATGCATATTCAATTGCGTCTCAGG
62	DegP (HtrA) amino acid (UniProtKB - P0C0V0) <i>E. coli</i> K12 (leader underlined at 1-26; DegP protein is 27-474; catalytic triad amino acids with/without leader at H131/105, D134/108, S236/210, in bold)	<u>MKKTTLALSALALSGLALSPLSATAAETSSATTAQQMPSLAPM</u> LEKVMPSVVSINVEGSTTVNTPRMPRNFQQFFGDDSPFCQEGSP FQSSPFCQGGQGGNGGGQQQKFMALGSGVIIDADKGYVVTNNHV VDNATVIKVQLSDGRKFDKAMVGKDPRSDIALIQIQNPKNLTAI KMADSDALRVGDYTVAINGNPFGLGGETVTSGIVSALGRSGLNAEN YENFIQTDAAINRGN SGG ALVNLNGELIGINTAILAPDGGNIGI GFAIPSNMVKNLTSQ MVEY GQVKRGELGIMGTELNSELAKAMKV DAQRGAFVSQVLPNSSAAKAGIKAGDVITSLNGKPISSFAALRA QVGTMFVGSKLTLGLLRDGKQVNVNLELQQSSQNQVDSSSIFNG IEGAEMSNKGKDQGVVNVNVKTGTAAQIGLKKGDVIGANQQA VKNIAELRKVLDSKPSVLALNIQRGDSTIYLLMQ
63	MepM amino acid (alternative name YebA) P0AFS9 <i>E. coli</i>	MQQIARSVLAFNNLPRPHRVMLGSLTVLTLAVAVWRPYVYHRD ATPIVKTIELEQNEIRSLLEPEASEPIDQAAQEDEAIPQDELDK IAGEAGVHEYVYSTGDTLSSILNQYGIDMGDITQLAAADKELRN LKIGQQLSWTLTADGELQRLTWEVSRRETRTYDRTAANGFKMTS EMQQGEWVNNLLKGTVGGSFVASARNAGLTSAEVSAVIKAMQWQ MDFRKLKKGDEFVLMSREMLDGKREQSQLLGVRLRSEGKDYYA IRAEDGKFYDRNGTGLAKGFLRFPTAKQFRISSNFNPRRTNPVT GRVAPHRGVDFAMPQGTVPVLSVGDGEVVVAKRSGAAGYYVAIRH GRSYTTRYMHLRKILVKPGQKVKRGDRIALSGNTGRSTGPHLHY EVWINQQAVNPLTAKLPRTEGLTGSDRREFLAQAKEIVPQLRFD
64	MepM2 WP_153670715.1 <i>E. coli</i>	MPRLAPLLALSLLLLAGGAQASYITRTLKNKVPVGGVAVVDLGP AASAPSARFDGKPVLVVKEQDNWLAIVGIPLTQKPGTAVLSQGG RTLFPFTVGSKKYPEQRITLKNTRQVNPNPADLKRIDRELAEQIK AYRSFSPTLPSNLILDKPVSGPLSSKFGVRRFFNGEERNPHAGL DFAVPAGTPIKTPANGKVILVGDYFFNGRTVFVDHGQGFISMFC HMSKIDVQVGQQLRRGEVVGVRVGTGRATGPHMHWNVSLNDARV DPA
65	MepM1	MTNEPTKAPPLYPKSHLLAASGIAALLSLALLVFPSSSEVEAKKT TLNLELESPAELKQQETTQADVREETTASPFQIDTAPAPTEE

	A0A0D1M539 <i>Pseudomonas Putida</i>	TAKTEPTPTAEPKDP SHREVTVARGDTLSTLFAKVGLPANVVH EVLASNKQAKQFSQLKHGQVLEIELDKDQGLASLHKSVDLETI RLTKGDKGYAFNREITKPVVRSAYVHGVIKSSLSASAQRAGLNH SLTMDMARIFGYDIDFAQDIRQGDEFDVIYEQKVVGKVVGN ILSARFTNRGKSFTAVRYTNKQGNSTSYTADGNSMRKAFIRTPV DFARISSRFSAGRKHPIILNKIRAHKGV DYAAPRGTPIKAAGDGK VLLAGRRGGYGNTV I IKHGNTYQTLYGHMQGFAKGIKTGGTVKQ GQVIGYIGTTGLSTGPHLHYEFQVNGVHVDPLGQKLPMADPIAK AERQRFLQQSQPLMARMEQEKATMLASAKR
66	MepM1 PA0667 <i>Pseudomonas aeruginosa</i>	MFPSSSEVEAKRTTLNLELESNTDRLLQEKDDLLPQSVTNSSDEG TPFAQVEGASDDNTAEQDSDKPGASVADADTKPVDPEWKTITVA SGDTLSTVFTKAGLSTSAMHDMLTSSKDAKRFTHLKVGQEVKLG LDPKGELQALRVKQSELETIGLDKTDKGYSFKREKAQIDLHTAY AHGRITSSLFVAGR NAGLPYNLVTSLSNIFGYDIDFALDLREGD EFDVIYEQHKVNGKQVATGNILAARFVNRGKTYTAVRYTNKQGN TSYYRADGSSMRKAFIRTPVDFARISSRFSLGRRHPIILNKIRAH KGV DYAAPIGTPIKATGDGKILEAGRKG GYGN AVVIQHGQRYRT IYGHMSRFAKGIRAGTSVKQGQIIIGYVGMTGLATGPHLHYEFQI NGRHVDPLSAKLPMADPLGGADRKR FMAQTQPMIARMDQEKKTL LALNKQR
67	MepM2 WP_098965471.1 <i>P. putida</i>	MPRFFAPLLLLCLTSFNAHADS YITRLLNKPVPGGVAVVDL GSA TQASKATYQGKPV LVVKEQNNWLAIVGVPLTVKPGSQQISSGGR NLPFTVGNKKYPEQHITLKN TQQVNP NPANLKRIEGELAEQIKA YRSFSPNTPSNLLLDKPVNGPLSSKFGVRRFFNGEERNPHAGLD FAVPAGTPIKTPAAGKVILTGN YFFNGNTV FVDHGGGFISM FCH MSKIDVKVGDQLARGAVVGKVGSTGRATGPHMHNISLNDARVD PAIFIGAFQP
68	MepM2 TUEID40_04881 <i>P. aeruginosa</i>	MPRTLAFVSTLLLAALFCA LPTQADS FIMRLLNKPVPGGVAVVDL GEEGPPPRAFYQGKPV LVVREEGRW IAVVGIP LSTKPGPQKLEVRAATGNHEERFSVGSKHYREQRITL KNKRQVNPLPEDLKRIERELAEQTAA YRRFSPGLPSNLMLDKPVDG PLSSPFG LRRFFNGEERNPHSGLD FAVPAGTPIKAPAAGKVILIGDYFFN GKTVFVDHGGGFISM FCHLSKIDVKLGQQVPRGGVLGKVGATGR ATGPHMHNVS LNDARVDPAIFIGAF

		QP
69	DegP amino acid RXF01250 <i>P. fluorescens</i>	MLKALRFFGWPLLAGVLIAMLI IQRYPQWVGLPTLDVNLQQAPQ TNTVVQGPVTYADAVVIAAPAVVNLYTTKVINKPAHPLFEDPQF RRYFGDNGPKQRRMESSLGSGVIMSPEGYILTNNHVTTGADQIV VALRDGRETARVVGSDPETDLAVLKIDLKLNLPATLGRSDGLR VGDVALAIGNPFGVGQTVTMGIISATGRNQLGLNSYEDFIQTDA AINPGNSGGALVDANGNLTGINTAIFSKSGGSQGIGFAIPVKLA MEVMKSIIEHGQVIRGWLGIEVQPLTKELAESFGLTGRPGIVVA GIFRDGPAQKAGLQLGDIVLSIDGAPAGDGRKSMNQVARIKPTD KVAIILVMRNGKEIKLSAEIGLRPPPATAPVKEEQ
70	HtpX <i>E. coli</i>	MMRIALFLLTNLAVMVVFGVLVLSLTGIQSSSVQGLMIMALLFGF GGSFVSLMSKWMALRSVGGEVIEQPRNERERWLNTVATQARQ AGIAMPQVAIYHAPDINAFATGARRDASLVAVSTGLLQNMSPDE AEAVIAHEISHIANGDMVTMTLIQGVVNTFVIFISRILAQLAAG FMGGNRDEGEESNGNPLIYFAVATVLELVFGILASIIITMWFSRH REFHADAGSAKLVGREKMIA ALQRLKTSYEPQEATSMALCINGKSKSLSELFMTHPPLDKRIE ALRTGEYLK
71	Tsp (Prc) <i>E. coli</i> GenBank M75634.1	MNMFFRLTALAGLLAIAGQTFAVEDITRADQIPVLKEETQHATV SERVTSRFTSRSHYRQFDLDQAFSAKIFDRYLNLLDYSHNVLLAS DVEQFAKKKTELGDELRSGLDVFYDLYNLAQKRRFERYQYALS VLEKPMDFGTGNDTYNLDLRSKAPWPKNELNALWDSKVKFDELS LKLTKGTDKEIRETLTRRYKFAIRRLAQTNSEDVFSLAMTAFAR EIDPHTNYLSPRNTEQFNTEMSLSLEGIGAVLQMDDDYTVINSM VAGGPAAKSKAISVGDKIVGVGQTGKPMVDVIGWRLDDVVALIK GPKGSKVRLEILPAGKGTKTRTVTLTRERIRLEDRAVKMSVKT GKEKVGVLDPGFYVGLTDDVKVQLQKLEKQNVSSVIIDLRNG GGALTEAVSLSGLFIPAGPIVQVRDNGKVRESDTDGQVFYKG PLVVLVDRFSASASEIFAAAMQDYGRALVVGEPTFGKGTVQQYR SLNRIYDQMLRPEWPALGVSQYTIQKFYRVNGGSTQRKGVTPDI IMPTGNEETETGEKFEEDNALPWDSIDAATYVKSGDLTAFEPELL KEHNARIAKDPEFQNMKDIA RFNAMKDKRNIVSLNYAVREKEN NEDDATRLARLNERFKREGKPELKKLDDLPKDYQEPDPYLDET V NIALDLAKLEKARPAEQPAPVK

72	MepS Q88MA5 <i>P. putida</i>	MPMLKRFAPLVPLALVTLLFGCAAQGPVSPQPDHTPITAQSAIN AKASSSSVFGEPEELATEDDLASFSGGKPYQLPVLADSILERM SLIGTRYRFGGTSEKSGFDCSGFIGYLFREEAGMTLPRSTREMI NVDAPKVARNKLPDGLFFSTNGRGRVSHAGIYLGDNQFIHSS SRRSGGVRIDSLGDRYWSKTFIEAKRALAMAPTNIARN
73	MepS MXH34301.1 <i>P. aeruginosa</i>	MVKSQPILRYILRVAPAIHAVVLLSACSSTSTARNMHSETHAVG SGDLSSLQASQDEFETMVRNLDVKSRLMDQYASWKGVRVRLGGS TRKGIDCSAFVQRTFREQFGLELPRSTSEQQETGKSISRTQLRT GDLVLFRAGSTGRHVGIYLGNNQFVHASTSSSGVTISSMDEPYWK KRYNEARRVLSRS
74	HslU example nucleic acid encoding SEQ ID NO: 37	ATGTCCATGACTCCCCGCGAAATCGTCCATGAACTCAATCGCCA TATCATCGGCCAGGACGATGCCAAGCGCGCCGTTGCCATTGCGC TGCGTAACCGCTGGCGCCGGATGCAACTGCCGGAAGAACTGCGC GTTGAAGTAACGCCCAAGAACATCCTGATGATCGGCCCCACCGG CGTGGGTAAAACCGAGATCGCCCGGCGCCTGGCCAACTGGCCA ATGCACCGTTCATCAAGGTCGAAGCGACCAAGTTCACCGAAGTC GGCTATGTGGGCCGCGATGTGAGTCGATCATTCGTGACCTGGC TGACGCCGCCCTGAAGATGCTGCGCGAACAGGAAGTAACCAAGG TCAGCCACCGCGCCGAAGACGCCGCTGAAGAGCGCATCCTCGAC GCCCTGTTGCCACCGGCACGCATGGGTTCACCAAGACGCCCGC ACCGGCTACCGATTCCAACACTCGCCAGCTGTTCCGCAAGCGCC TGCGTGAAGGCCAGCTGGATGACAAGGAAATCGAGATCGAAGTG GCTGAAGTGTCCGGCGTGGATATTTCTGCCCCGCTGGCATGGA AGAAATGACCAGCCAGCTGCAGAACCTGTTGCGCAACATGGGCA AGGGCAAGAAGAAAAGCCGCAAGCTCAAGGTGAAAGAGGCGCTC AAGCTCGTGCGGACGAAGAAGCCGGGCGCCTGGTCAATGAGGA AGAACTCAAGGCCAAGGCCCTGGAAGCGGTGAGCAACATGGCA TCGTGTTTATCGACGAGATCGACAAAAGTGCCCAAGCGAGGCAAC TCAGGCGGCGTGGATGTGTCCCGCAAGGCGTGCAGCGCGATTT GCTGCCGCTGATCGAGGGCTGCACGGTCAACACCAAGCTGGGCA TGGTCAAGACTGACCACATCCTGTTTATCGCTTCCGGTGCTTTC CACCTGAGCAAGCCCAGCGACCTGGTGCCCCGAGCTGCAAGGCCG CTTGCCGATTTCGGGTGGAGCTCAAGGCGCTGACGCCGGGCGACT TCGAGCGCATCCTCAGCGAGCCGCATGCCTCGCTCACCAGAGCAG TACCGCGAGTTGCTGAAAACCGAAGGGCTGGGTATCGAATTCCA

		GGCAGACGGGATCAAGCGCCTGGCGGAGATCGCCTGGCAGGTCA ACGAGAAGACCGAGAACATCGGTGCCCCGTCGCCTGCATACCTTG CTTGAGCGCCTGCTGGAGGAAGTGTCTTCAGTGCCGGCGACAT GGCCGGTGCGCAGAATGGCGAAGCGATCAAGATCGATGCTGATT ACGTCAACAGCCACTTGGGCGAATTGGCGCAGAACGAAGATCTG TCTCGTTATATCCTGTAA
75	HslV example nucleic acid encoding SEQ ID NO: 38	ATGACCACCATCGTTTCAGTACGTGCCCACGGCAAAGTTGTCAT GGGCGGCGACGGCCAGGTTTCCCTGGGCAACACCGTGATGAAAG GCAACGCCAAGAAAGTGCGCCGCTGTACCACGGCCAGGTGCTT GCCGGCTTCGCAGGCGCAACCGCCGACGCCTTTACCCTGTTCGA GCGTTTCGAAGGCCAGCTTGAGAAACACCAGGGCCACCTGGTGC GCGCCGCTGTGGAAGTAGCCAAAGAATGGCGCACCGACCGCTCC CTCAGCCGCTGGAGGCCATGCTCGCGGTTGCGAACAAAGACGC TTCCCTGATCATCACTGGCAACGGCGACGTGGTTGAACCCGAGC ATGGCCTGATCGCCATGGGTTCCGGCGGCGGCTACGCCACAGGT GCGGCCAGCGCGCTGTTGAAGAAAACCGACCTGTCGGCCCCGTGA AATCGTCGAGACCGCCCTGGGTATCGCTGGCGATATCTGCGTGT TCACCAACCACAACCAGACCATTGAGGAGCAGGACCTCGCCGAG TAA
76	Putative cytoplasmic disulfide isomerase DsbA amino acid (RXF07017.2) <i>P. fluorescens</i>	MSKTLFFFDLGSPTAYLAYTRLPALCAETGAQVVYQPMLLGGV FKATGNASPITVPAKGRYMLDDLARYAKRYNVPLRFNPFPINT LLLMLRAVTGIQIHQPERFLDFIGCLFRALWVEGRHLGDPEVVAN VLTEQGFDPQVLALSNDAAVKDALDKTEQAIRGVFGAPSF VGNQLFFGQDRDLDFVREALS
77	Disulfide-bond isomerase DsbA homolog amino acid (RXF01002.1)	MRNLILSAALVTASLFGMTAQAAADVPLEAGKTYVELANPVPVAV PGKIEVVVELFWYGCPHCYAFEPTINPWAEKLPKDVNFRRIPAMF GGPWDAGHQLFLTLEAMGVEHKVHNAVFEAIQKQKRLTKPDEM ADFVATQGVDDKDFLATFNSFAIQGQIKQAKELAQKYGVQGVPT LIVNGKYRFDLGSTGGPEATLNVDQLIAKERAANK
78	Disulfide-bond isomerase DsbB amino acid (RXF03204.1)	MIDDMRLGRERRFLVLLGIICLALIGGALYMQVVLGEAPCPLCI LQRYALLLIALFAFIGAAMRTKGALTFFEGLVLSALGGVAAAG HHVYTQFFPQVSCGIDVLQPIVDDLPLAKVFPLGFQVDGFCSTP YPPILGLSLAQWALVAFVLTAILVPLCIYRNRHPKA

79	Disulfide-bond isomerase DsbC example nucleic acid (RXF03307; PROKKA_03627) encoding SEQ ID NO: 60	ATGCGCTTGACCCAGATTATTGCCGCCGCAGCCATTGCGTTGGT TTCCACCTTTGCGCTCGCCGATGATGCGGCCGAGCAGACCATCC GCAAGAGCCTGGCCAACCTGGCGCTCGACACGCCTATCGAAAAGC ATTAGCGCCAGCCCCATGGCCGGCCTGTACGAAGTCAAGCTCAA GGGCAGCCGCGTGCTGTACGCCAGTGCCGATGGCCAGTACATCG TCCAGGGCTACCTGTTCCAGCTCAAGGACGGCAAGCCGGTCAAC CTGACCGAGAAGGCCGAGCGCCTGGGCGTGTTCCAAGCTGATCAA CGGCATCCCGGTGGCTGAAACCGTGGTTTACCCGGCCATTGGCG AAACCAAGACCCACATCACCGTGTTACCGACACCACCTGCCCG TACTGCCACAAGCTGCACGCTGAAATCCCGGCACTGAACAAGCT GGGCGTGGAAGTGCGCTACGTGCGGTTCGCGGCCAGGCCTGG GTTGCGCCGGGTGACGAGCAGTTGCAAGCCGTATGGTGTTCGGCC GACAAAAGGCGGCCATGGACAAGATGGTCGACGGCAAGGAAAT CAAATCGGCCAAATGCGCCAACCCGGTTTCCAAGCAGTTCGCCC TGGGCCAGTCCATTGGTGTGAACGGTACACCGGCCATCGTTTTG GCCGACGGCCAGGTGATTCCGGGCTACCAGCCGGCGCCGCAAGT TGCCAAACTGGCACTGGGTGCCAAG
80	Disulfide-bond isomerase DsbD amino acid (RXF04886.2)	MRHLFTFLVLVLFAGFAQAAPGSPFETKPDFLPVGKAFATSERL ESGETQLFWQIADGYLYQQRMKFDGLAEKPVLPGEAHSDEFF GEQQVYRQGLEVKIPAGTTGQVKLGWQGCADAGLCYPPQSITVD LGGNPAVAATAQAQDQSLASGLQQRSLGWSLLVFFGLGLLLAFA PCSLPMLPILAGLVVSGASPRRGFALAGSYVVCMLVYAALGV MAALLGANLAALLQTPWILGSFAALFVLLALPMFGFFELQLPAF LRDRLDNVSRQQSGGSLVGAGVLGALSGLLVGPCMTAPLAGALL YIAQSGNALHGGLILFAMGIGIGIPLLLLTVGNRFLPKPGTWM NVLKGI FGFLFLGTAVLMIRPVVGDSLWIGLWGALALVMAYCGW ALARESGLAAKVFGAGSLVLGLWGAVLVGAAGGSDELWQPLKV YSGSRVADAPSAHDAFTTVSDPAVLQSQLD SAKAQGWVLLDYY ADWCVSCKIMEKQVFGKPEVMDALKDVRLRLDVTADNAASREL LGRYKVPGPSFVWIGPDGEERRAQRITGEVDAAAFQRWTQTR DAR
81	Disulfide-bond isomerase DsbG amino acid (RXF04890.2)	MPRLRHLLTLLPLTLAAALQAEDLPAPIKQIEAKGAKIIGKFD APSGLTGYAAQYQNRGMALYLTADGKNVIAGNLYDAQNDLSTA PLEKL VYAPMAKEVWAKMENS SWIQDGDKNAPRTIYLFSDPNCP YCNMFWEQARPWVKAGKVQLRHIMVGI IREDS PGKSAALLAAKD

		PQKALQDHEAAGKSGSKLKALEKIPAEVEAKLDANMKLMDELELS ATPAIFYLDDKGGLQQQQGAPSPDKLVKILGPK
82	PDIA6 amino acid UniProt Q922R8 mouse	MARLVGLVLSCTFFLAVSGLYSSDDVIELTPSNFNREVIQSDG LWLVEFYAPWCGHCQRLTPEWKKAAATALKDVVKVGAVNADKHQS LGGQYGVQGFPITIKIFGANKNKPEDYQGGRTGEAIVDAALSALR QLVKDRLGGRSGGYSSGKQGRGDSSSKDVVELTDDTFDKNVLD SEDVMMVEFYAPWCGHCKNLEPEWAAAAATEVKEQTKGKVKLAHV DATVNQVLASRYGIKGFPTI KIFQKGESPVVDYDGGRTSRDIVSRALDLFSDNAPPELLEIINE DIAKKTCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKYK KKMWGLWTEAGAQYELLENALGIGGFGYPAMAAINARKMKFALL KGSFSEQGINEFLRELSFGRGSTAPVGGGSFPTITPREPWDGKD GELPVEDDIDLSDVELDDLEKDEL
83	PDIA6 amino acid Q3TML0 mouse	MRVIGMARLVGLVLSCTFFLAVSGLYSSDDVIELTPSNFNREV IQSDGLWLVEFYAPWCGHCQRLTPEWKKAAATALKDVVKVGAVNA DKHQSLGGQYGVQGFPITIKIFGANKNKPEDYQGGRTGEAIVDAA LSALRQLVKDRLGGRSGGYSSGKQGRGDSSSKDVVELTDDTFD KNVLDSEDVMMVEFYAPWCGHCKNLEPEWAAAAATEVKEQTKGKV KLAHV DATMNQVLASRYGIK GFPTIKIFQKGESPVVDYDGGRTSRDIVSRALDLFSDNAPPELL EIIINEDIAKKTCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKL ADKYKKMWGLWTEAGAQYELLENALGIGGFGYPAMAAINARKM KFALLKGSFSEQGINEFLRELSFGRGSTAPVGGGSFPTITPREP WDGKD GELPVEDDIDLSDVELDDLEKDEL
84	PDIA6 amino acid Q63081 rat	MARLVGLVLSCTFFLAVSALYSSDDVIELTPSNFNREVIQSDS LWLVEFYAPWCGHCQRLTPEWKKAAASALKDVVKVGAVNADKHQS LGGQYGVQGFPITIKIFGANKNKPEDYQGGRTGEAIVDAALSALR QLVKDRLGGRSGGYSSGKQGRGDSSSKDVVELTDDTFDKNVLD SEDVMMVEFYAPWCGHCKNLEPEWAAAAATEVKEQTKGKVKLAHV DATVNQVLASRYGIKGFPTI KIFQKGESPVVDYDGGRTSRDIVSRALDLFSDNAPPELLEIINE DIAKKTCEEHQLCVVAVLPHILDTGATGRNSYLEVLLKLADKYK KKMWGLWTEAGAQYELLENALGIGGFGYPAMAAINARKMKFALL KGSFSEQGINEFLRELSFGRGSTAPVGGGSFPNITPREPWDGKD GELPVEDDIDLSDVELDDLEKDEL

85	PDIA6 amino acid A0A5F5PFG7 horse	MKPAINGVLFFVSPGLMSCTLFLAVNGLYSSSDDVIELTPSNFN REVIQSDSLWLVEFYAPWCGHCQRLTPEWKKVATALKD VVKVGA VDADKHQSLGGQYGVQGFPTIKIFGANKNRPEDYQGGRSGEAI VDAALSALRQLVKDRLGGRSGGYSSGKQGRSESSSKKDVIELTDD SFDKNVLDSEDVMMVEFYAPWCGHCKNLEPEWAAAAATEVKEQTK GKVKLA AVDATVNQVLASRY GIRGFPTIKIFQKGESPVVDYDGGRTSRDIISRALDLFSDNAPPP ELLEIINEDI AKKTCEEHQLCVVAVLPHILDTGAAGRNSYLEVL LKLADKYKKMMWGLWTEAGAQSELETALGIGGGFGYPAMAAINA RKMKFALLKGSFSEQGINEFLRELSFGRGSTAPVGGGAFFAIST REPWDGKD GELPVEDDIDLSDVELDDLEKDEL
86	PDIA6 amino acid A0A5K1UH01 pig	MARLVLGLMSCTLFVAVNGLYSSSDDVIELTPSNFNREVIQSDS LWLVEFYAPWCGHCQRLTPEWKKVATALKD VVKVGA VDADKHQ SLGGQYGVQGFPTIKIFGSNKNRPEDYQGGRSGEAI VDAALSALR QLVKDRLGGRSGGYSSGKQGRSESGK KDVIELTDDTFDKNVLD SEDVMMVEFYAPWCGHCKNLEPEWAAAAATEVKEQTKGKVKLA AV DATVNQVLASRYGIRGFPTI KIFQKGESPVVDYDGGRTSRDIVTRALDLFSDNAPPPELLEIISE DVAKKSCCEEHQLCVVAVLPHILDTGAAGRNSYLEVLLKLADKYK KKMMWGLWTEAGAQTELEHALGIGGGFGYPAMAAINARKMKFALL KGSFSEQGINEFLRELSFGRGSTAPVGGGAFFAISTREPWDGKD GEVSPATREPGDGKDGQASPATREPWDGKDQASPATREPGDGK DGEASPAEPRGQDASRLWLSFLASLGPEAGCEPGLCIRAAPRAG PAVAPPGPRGLLLSSSSAPLPPATPQAKAPGSCSPGHSPQAERF STWREAQRGHFEVSLDSRTLPSGLERPTSVAPGVCPRDDGRS
87	PDIA6 amino acid P38660 Golden hamster	MARLGFGLVSCTFFLAASGLYSSSDDVIELTPSNFNREVIQSNS LWLVEFYAPWCGHCQRLTPEWKKAAATALKD VVKVGA VDADKHQ SLGGQYGVQGFPTIKIFGANKNKPEDYQGGRSGEAI VDAALSALR QLVKDRLSGRSGGYSSGKQGRGDSSSKKDVIELTDDTFDKNVLD SDDVMMVEFYAPWCGHCKNLEPEWATAATEVKEQTKGKVKLA AV DATVNQVLANRYGIRGFPTI KIFQKGEAPVDYDGGRTSRDIVSRALDLFSDNAPPPELLEIINE DVAKKMCEEHQLCVVAVLPHILDTGAARNSTYLEILLKLADKYKK KMWGLWTEAGAQSELENALGIGGGFGYPAMARINARKMKFALLK

		GSFSEQGINEFLRELSFGRASTAPVGGGSFPAITAREPWDGRDG ELPVEDDIDLSDVELDDLEKDEL
88	PDIA6 amino acid Q5R6T1 Pongo abelii (orangutan)	MALLVLGLVSCAFFLEVNGLYSSDDVIELTPSNFNREVIQSDS LWLVEFYAPWCGHCQRLTPEWKKAAATALKDVVKVGAVDADKHHS LGGQYGVQGFPITIKIFGSNKNRPEDYQGGRTGEAIVDAALSALR QLVKDRLGGQSGGYSSGKQGRSDSSSKKDVIELTDDSFDKNVLD SEDVWMVEFYAPWCGHCCKNLEPEWAAAASEVKEQTKGKVKLA AV DATVNQVLASRYGIRGFPTI KIFQKGESPVVDYDGGRTSRSDIVSRALDLFSDNAPPELLEIISE DIAKRTCEEHQLCVSVLPHILDTGAAGRNSYLEVLLKLADKYK KKMWGLWTEAGAQSELETALGIGGFYPAMAAINARKMKFALL KGSFSEQGINEFLRELSFGRGSTAPVGGGAFTIVEREPWDGRD GELPVEDDIDLSDVELDDLGKDEL
89	PDIA6 amino acid U3JW38 Collared flycatcher	MRESHKCSTGQLMSLLFLVGTVSCFLAVNGLYSASDDVIELT PTNFNKEVIQSESLWLVEFYAPWCGHCQRLTPEWKKAAATALKGV VKVGAVDADKHQSLGGQYGVRGFPITIKIFGANKNKAEDYQGGRT SDAIVDAALSALRSLVKERLSGRSGGYSSGKQSRGSGGDKKDV IELTDDSFDKNVINSDDVWMVEFYAPWCGHCCKNLEPEWAAAAATE VKEQTKGKVKLA AVDATVNQVLASRYGIRGFPTIKIFQKGEDPV DYDGGRTSRSDIVSRALDLFSDNAPPELLEIISEDVLKSTCDAH QLCIISVLPILDTGASGRNSYLDVMLKMAEKYKKMWGLWTE AGAQPDLLESSLGIGGFYPAMAAVNARKMKFALLKGSFSEQGIN EFLRELSVGRGSTAPVGGGAFPKIHSVEPWDGKDGE L PVEDDID LSDVDLDDFGKDEL
90	Protein disulfide- isomerase amino acid P55059.1 Human	MHKAQKFALGLLAAA AVATASDVVQLKKDTFDDFIKTNDLVLA E FFAPWCGHCKALAPEYEEAATTLKEKNIKLAKVDCTEETDLCQQ HGVEGYPTLKVFRGLDNVSPYKGQRKAAAITSYM IKQSLPAVSE VTKDNLEE FKKADKAVLVAYVDASDKASSEVFTQVAEKL RDNYP FGSSDAALAEAGVKAPAI VLYKDFDEGKAVFSEKFEVEAIEK FAKTGATPLIGEIGPETYSYMSAGIPLAYIFAETAERKELSD KLKPIAEAQRGVINFGTIDAKAFGAHAGNLNLKTDKFP AFAIQE VAKNQKFPFDQEKEITFEAIKAFVDDFVAGKIEPSIKSEPIPEK QEGPVTVVVAKNYNEIVLDDTKDVLIEFYAPWCGHCKALAPKYE ELGALYAKSEFKDRVVIKVDATANDVPDEIQGFPTIKLYPAGA

		KGQPVITYSGSRTVEDLIKFIENGKYKAAISEDAAETSSATETT TETATKSEEAAKETATEHDEL
91	Protein disulfide- isomerase amino acid O13811 <i>Schizosaccharomyce s pombe</i> (strain 972 / ATCC 24843)	MRLPLLSFVIFALFALVFASGVVELQSLNELENTIRASKKGALI EFYATWCGHCKSLAPVYEELGALFEDHNDVLIGKIDADTHSDVA DKYHITGFPTLIWFPPDGSEPVQYSNARDVDSLQTFVSEKTGIK KRKIVLPSNVVELDSLNFDDKVMDDKKDVLVEFYADWCGYCKRL APTYETLKGKVFKNPNVEIVKINADVADIGRLHEVASFPTIKF FPKDDDKDKPELYEGDRSLESLEIYINKKSGTQQRSPDGTLLSTAG RIPTFDEFAAEFLDMSNAAKEVVLKVKQLALEDSSRWTKYYKK VFEKILNDENVWHKEAKRLSKLLRQKSIALASADDFKTRLNINL SFLPGNH
92	Protein disulfide- isomerase amino acid A5LHW0 <i>Haemaphysalis longi cornis</i>	MATALLAVLAALSPMALAMYGPHTTEVVDLSPANFKNRVDSDEV WIVEFYAPWCGHCQSFAPEYTKAAAALKGIVKVGAVDADKDKSL GGQYGVRGFPTVKIFGANKHNPTDYSGPRTADGVASAALQEARK VVDQRLGRKTSGGSSGGKSDVVELDESNFEELVLKSDDLWLVEF FAPWCGHCKNLAPHWAKAATELKGKVKLGAVDATVHQGLASQFD VKGYPYTIKFFPGGKKDRHSAXEYNGGRTADDIVQWGLDKAAESA PAPELHQVTSPSVLKDACEESQLCVVSVLPHIYDCQSECRQGYL DVLKRLGEKYKRNWGWLWSEALAQPKEEAELEIGGFGYPALAV LNSRKMKYSLLRGSFSDYDGINEFLRELAVGRGSSVPVKGAKLPE VQTVEPWDGKDAKLEEPEDIDLSDVELEPEEPGKKHVEL
93	Protein disulfide- isomerase amino acid Q5UAH0 <i>Plasmodium chabau di chabaudi</i>	MNSKYFSFLLFLIPFLFQNCVRSHEDLFNEHVTSIHDGELTNFI TKNDIVLVMFYAPWCGHCKRLIPEYNDAAIMLAEEKSEIKLASV DATIERGLSQEYGITGYPTMILFNKKNRINYGGGRTAQITVDWI LQMTGPVSTEITGNIEDVLKEKNINVAFYIEYTSDEHLEFKKN EVGDKNREIAKYFMKKNDKHNKIYCYRKDEKTVEYDEKTPLSDF ITIESFPLFGEINTENYRFY AESPKELVVVCATIEQYNEIKEEVRLAAAELRNKTHFVLLNIPE YADHAKASLGINEFPGLAYQSSEGRYVLTNPKQSLKNHKDIITF FKDVEAGKIEKSLKSEPIPEEDKDAPVKVVVGNFIDVVLKSGK DVLIEIYAPWCGHCKKLEPVYEELGRKLKDYDHIIVAKMDGTIN ETALKEFEWSGFPTIFFVKAGSKIPLPYEGERSLKGFVDFLNKH STKTPITIDGVSQSDDGASE EL

94	Protein disulfide-isomerase amino acid XP_024705795.1 <i>Aspergillus steynii</i> I BT 23096	MRSFTPWVLGLLGASAVVSAGDAQADVPSDVKSLTQDTFNDFIK EHDLVLAEFFAPWCGHCKALAPKYEAAASQLKDKNIPLVKIDCT EEEELCRDQGVGYPTLKI FRGVDSSKPYQGARQTESLVSYMIK QSLPAVSSVNEENLEDTKTMDKIVVIGYFSSDDQAANDAFNALA EAQRDNLYLFAATDDAAIAKAEGVEQPSLVLYKDFDEKKAIYTGE IEQDAVLTWVKTASTPLVGEIGPETYSSYITAGIPLAYIFAETS EEREKFTEDFKPIAEKHKGLINIATIDAKMFGAHAGNLNLDPQT FPAFAIQDPEKKAKYPYDQSKEITAKDVGKFIQDVLGGKVEPSI KSEPIPESQEGPVTVVVAHSYKELVVDNEKDVLLIFYAPWCGHC KALAPKYEELASLYADVDPDLASKVTIAKIDATANDVPDSITGFP TIKLYPAGGKDAPVEYAGSRTVEDLVNFVKENGQHKVDALANTQ EGGDATESPSASSETTEAPAATDDKADHDEL
95	Protein disulfide-isomerase amino acid PGH31646.1 <i>Emmonsia crescens</i>	MRQFRDFAFGLAALGLTALASATEAEAESDVHVLKKDTFNDFMN SHDLVLAEFYAPWCGHCKALAPEYEVAATELKEKNIHLAKIDCT EEADLCQEHGVEGYPTLKI FRGLENVKPYTGPRKSGPIASFMVK QSLPPVTTVTADNIEDVKTLDKIVVIGYFAEDDKASNETFTAVA EALRDDYLFAGTNDAKLAAAEDVKQPAIVLYKEFDERKAVFKNK FVQDDISKFVKTASIPLVGEVGPDTYAGYMASGLPLAYVFAETP EEREFEAAMLKPIAQKQKGSINIATIDAKAFGAHAGNLNLDPEK FPAFAIQDTTNNKKYPFDQTKKITHDDIAKFVQDVLGKVEPSI KSEPIPESQDAAVTVVVAHSFQEIVIDNDKDVLEVEYAPWCGHC KALAPKYEQLGQLYADVPEFASKVTIAKIDATANDVPEDIQGF TIKLYAAGSKGSPVDYDGSRTIEDLAKFVRDNGKHGVDAYVAEK VVEDGGDVTNSPAAASPSSTAADKESETSSSDAEETAEAPRHE EL
97	Protein disulfide-isomerase Pdi1 amino acid KEY81650.1 <i>Aspergillus fumigatus</i> var. RP- 2014	MRSFAPLVLSLLGASAVASADATADTTSDVVSLTKDSFKDFMKE HDLVLAEFYAPWCGHCKALAPKYEAAATELKGKNIPLVKVDCTE EEDLCKENGVEGYPTLKI FRGPDSSKPYQGARQADSIVSYMIKQ SLPAVSAVTEENLEEIKTMDKIVVIGYFASDDKAANDVFTSFAE SQRDNYLFAATSDSAIAKAEGVKQPSIVLYKDFDEKKAVYDGAI EQEAILSWVKTASTPLVGEIGPETYSSYITAGIPLAYIFAETKE ERDQYAEDFKPVAEKHKGAINIATIDAKMFGAHAGNLNLDPQTF PAFAIQDPEKNAKYPYDQSREFNAKEIGKFIQDVLGKVEPSIK SEPIPETQEGPVTVVVAHSYQDIVINNDKDVLLIFYAPWCGHCK ALAPKYEELAALYAGDFKDKVTIAKIDATANDVPDSITGFPTIK

		LYPAGAKDSPVEYSGSRTVEDLANFIKENGKYKVDALVAASEKV EEGPDVTASPSATSTEAEAPAAATGDEKGDHDEL
98	Protein disulfide- isomerase FrnE amino acid (RXF08657.2) <i>P. fluorescens</i>	MSTPLKIDFVSDVSCPWCIIIGLRGLTEALDQLGSEVQAEIHFQP FELNPNMPAEGQNIVEHITEKYGSTAEESQANRARIRDMGAALG FAFRTDGQSRIYNTFDAHRLHGWAGLEGLQYNLKEALFKAYFSD GQDPSDHATLAIIAESVGLDLARAAEILASDEYAAEVREQEQLW VSRGVSSVPTIVFNDQYAVSGGQPAAEFVGAI RQI INESKS

*Public database numbers identify sequence information that is incorporated by reference herein.

CLAIMS

WHAT IS CLAIMED IS:

1. A recombinant gram-negative bacterial host cell for recombinant protein expression, wherein the host cell is:
 - (a) deficient in a first protease activity, wherein the first protease activity is tail-specific protease activity, wherein the deficient first protease activity results from a mutation in at least one gene encoding a tail-specific protease;
 - (b) deficient in a second protease activity, wherein the second protease activity is murein DD-endopeptidase activity, wherein the deficient second protease activity results from a mutation in at least one gene encoding a murein DD-endopeptidase.
2. The recombinant gram-negative bacterial host cell of claim 1, wherein the host cell further:
 - (c) is deficient in at least one additional protease activity, wherein the deficient additional protease activity results from a mutation in at least one gene encoding an additional protease, wherein the additional protease is different from the proteases of (a) and (b);
 - (d) is deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor;
 - (e) overexpresses one or more inactivated protease;
 - (f) overexpresses one or more folding modulator; or
 - (g) any combination of (c), (d), (e) and (f).
3. The recombinant gram-negative bacterial host cell of claim 1 or 2, wherein the deficient tail-specific protease activity results from a mutation in a gene encoding one or more of:
 - (i) a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33, a homologue of SEQ ID NO: 33, or a Prc1 tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 33;
 - (ii) a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35, a homologue of SEQ ID NO: 35, or a Prc2 tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 35; or
 - (iii) a Tsp tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 71, a homologue of SEQ ID NO: 71, or an Tsp tail-specific protease related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 71.

4. The recombinant gram-negative bacterial host cell of any one of claims 1-3, wherein the deficient murein DD-endopeptidase activity results from a mutation in a gene encoding one or more of:

- (i) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 1, a homologue of SEQ ID NO: 1, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 1;
- (ii) a MepM murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 63, a homologue of SEQ ID NO: 63, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 63;
- (iii) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 65, a homologue of SEQ ID NO: 65, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 65; and
- (iv) a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 66, a homologue of SEQ ID NO: 66, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 66.

5. The recombinant gram-negative bacterial host cell of any one of claims 2-4, wherein: the host cell of (c) is deficient in 1 to 10 different additional protease activities; the host cell of (d) is deficient in 1-5 different autolytic factor activities; the host cell of (e) overexpresses 1 to 10 different inactivated proteases, wherein each inactivated protease is different; the host cell of (f) overexpresses 1-10 different folding modulators, or any combination thereof.

6. The recombinant gram-negative bacterial host cell of any one of claims 2-5, wherein:

the one or more deficient additional protease activity of (c) results from a mutation of at least one gene encoding an additional protease independently selected from: a serralyisin precursor, a membrane-localized protease, a murein L,D transpeptidase, a hemolysin precursor, a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor, a periplasmic serine endoprotease, an AAA+ family proteolytic machine, and a murein DD-endopeptidase different from that of (a);

the one or more deficient autolytic factor activity of (d) results from a mutation of at least one gene encoding an autolytic factor independently selected from: an S-type pyocin, a linear gramicidin synthase subunit D, a hemolysin precursor, a leukotoxin, and a porin;

the one or more inactivated protease of (e) is a mutant periplasmic serine endoprotease;
and

the one or more folding modulator of (f) is a disulfide isomerase.

7. The recombinant gram-negative bacterial host cell of claim 6, wherein:

the serralysin precursor is selected from:

a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a
homologue of the serralysin precursor having the amino acid sequence set forth as SEQ
ID NO: 9; a serralysin precursor related protein having at least 60% similarity or at least
60% identity to the amino acid sequence set forth as SEQ ID NO: 9;

a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 47; a
homologue of the serralysin precursor having the amino acid sequence set forth as SEQ
ID NO: 47; and a serralysin precursor related protein having at least 60% similarity or at
least 60% identity to the amino acid sequence set forth as SEQ ID NO: 47;

the membrane-localized protease is an HtpX having the amino acid sequence set forth as
SEQ ID NO: 39, a homologue of the HtpX having the amino acid sequence set forth as
SEQ ID NO: 39, or an HtpX related protein having at least 60% similarity or at least 60%
identity to the amino acid sequence set forth as SEQ ID NO: 39;

the murein L,D transpeptidase is a murein L,D transpeptidase having the amino acid
sequence set forth as SEQ ID NO: 41, a homologue of the murein L,D transpeptidase
having the amino acid sequence set forth as SEQ ID NO: 41, or a murein L,D
transpeptidase related protein having at least 60% similarity or at least 60% identity to the
amino acid sequence set forth as SEQ ID NO: 41;

the hemolysin precursor is a hemolysin precursor having the amino acid sequence set
forth as SEQ ID NO: 43, a homologue of the hemolysin precursor having the amino acid
sequence set forth as SEQ ID NO: 43, or a hemolysin precursor related protein having at
least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ
ID NO: 43;

the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor is a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45, a homologue of the D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor having the amino acid sequence set forth as SEQ ID NO: 45, or a D-alanyl-D-alanine carboxypeptidase/endopeptidase AmpH precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 45;

the periplasmic serine endoprotease is selected from:

a DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a homologue of the DegP2 having the amino acid sequence set forth as SEQ ID NO: 31; a DegP2 related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 31;

a DegP having the amino acid sequence set forth as SEQ ID NO: 69; a homologue of the DegP having the amino acid sequence set forth as SEQ ID NO: 69; a DegP related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 69;

a DegP having the amino acid sequence set forth as SEQ ID NO: 62; a homologue of the DegP having the amino acid sequence set forth as SEQ ID NO: 62; and a DegP related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 62;

the AAA+ family proteolytic machine comprises: an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38;

the murein DD-endopeptidase is selected from:

a *P. fluorescens* MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 3; a homologue of the MepM2 protease having the amino acid sequence set forth as

SEQ ID NO: 3; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 3;

a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 64; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 64;

a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 67; a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 67;

a MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68; a homologue of the MepM2 protease having the amino acid sequence set forth as SEQ ID NO: 68; and

a MepM2-related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 68;

the S-type Pyocin is an S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49, a homologue of the S-type Pyocin having the amino acid sequence set forth as SEQ ID NO: 49, or an S-type Pyocin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 49;

the linear gramicidin synthase is a linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51, a homologue of the linear gramicidin synthase having the amino acid sequence set forth as SEQ ID NO: 51, or a linear gramicidin synthase related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 51;

the leukotoxin is a leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53, a homologue of the leukotoxin having the amino acid sequence set forth as SEQ ID NO: 53, or a leukotoxin related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 53;

the ShlB hemolysin transporter is an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55, a homologue of the an ShlB hemolysin transporter having the amino acid sequence set forth as SEQ ID NO: 55, or an ShlB hemolysin

transporter related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 55;

each of the one or more overexpressed inactivated proteases is independently selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2-related protein comprising an amino acid substitution or disruption of a DegP2 having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 69; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA comprising an amino acid substitution or disruption of a homologue of the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP/HtrA-related protein comprising an amino acid substitution or disruption of a DegP having at least 60% similarity or at least 35% identity to amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of an amino acid at a position corresponding to any one of 131 (His), 134 (Asp) and 236 (Ser) (SEQ ID NO: 62, numbering including leader sequence 1-26), or respective positions 105, 108, and 210, when excluding the leader sequence; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr S210A; an inactivated DegP, DegP-related protein, or DegP homologue having an amino acid substitution corresponding to *E. coli* Htr H105R; and an inactivated DegP, DegP-related protein, or DegP homologue having a substitution or disruption of any one or more amino acid at a position corresponding to any one of: 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234 of SEQ ID NO: 31; and

each of the one or more folding modulators is independently selected from: a disulfide bond isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 60, 76, 77, 78, 80, and 81; a homologue of a disulfide bond isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 60, 76, 77, 78, 80, and 81; a disulfide bond isomerase-related protein having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as any one of SEQ ID NOS: 60, 76, 77, 78, 80, and 81; a protein disulfide isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 27 and 82-98; a homologue of a protein disulfide isomerase having an amino acid sequence set forth as any one of SEQ ID NOS: 27 and 82-98; and a protein disulfide isomerase-related protein having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NOS: 27 and 82-98.

8. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the mutation is in a coding sequence or noncoding sequence of the corresponding gene, and wherein the mutation is independently selected from: (i) a complete gene deletion, (ii) a partial gene deletion, (iii) a missense mutation, (iv) a nonsense mutation, (v) a frameshift mutation, (vi) an insertion, and (vii) any combination of (ii), (iii), (iv), (v) and (vi).

9. The recombinant gram-negative bacterial host cell of claim 8, wherein the missense mutation of (iii) results in a conservative or non-conservative amino acid substitution.

10. The recombinant gram-negative bacterial host cell of claim 8 or 9, wherein the noncoding sequence is a regulatory sequence.

11. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is the activity of: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 protease amino acid sequence set forth as SEQ ID NO: 5.

12. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is: a MepS2 having the amino acid sequence set forth as SEQ ID NO: 7; a homologue of the MepS2 having the amino acid sequence set forth as SEQ ID

NO: 7; or a MepS2 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS2 protease amino acid sequence set forth as SEQ ID NO: 7.

13. The recombinant gram-negative bacterial host cell of claim 11 or claim 12, wherein the gram-negative bacterial host cell is a *Pseudomonad*.

14. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the gram-negative bacterial host cell is a *Pseudomonad*, and the deficient first protease activity results from a mutation of a coding sequence and/or noncoding sequence of a gene encoding *P. fluorescens* Prc1 and/or a mutation of a coding sequence and/or noncoding sequence of a gene encoding *P. fluorescens* Prc2.

15. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the second protease activity is deficient due to a mutation that results in a conservative or non-conservative substitution in an active site amino acid or an allosteric site amino acid of a protease having the second protease activity.

16. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the deficient second protease activity results from at least one mutation of the second protease gene, wherein the mutation results in a disruption of the amino acid sequence at a position corresponding to: (i) any one or more of residues 134 to 145 of SEQ ID NO: 1; (ii) any one or more of residues 319 to 411 of SEQ ID NO: 1; (iii) one or more of residues any 361 to 378 of SEQ ID NO: 1; (iv) any one or more residue selected from 248, 319, 330, 332, 334, 337, 378, 410, and 411 of SEQ ID NO: 1; or any combination of (i), (ii), (iii), and (iv).

17. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the bacterial host cell is *Pseudomonas fluorescens*, wherein the deficient second protease activity results from a gene mutation that results in an amino acid substitution of SEQ ID NO: 1 selected from: Y248stop, G332S, D334N, A337T, H411Y, P410L, and any conservative or non-conservative amino acid substitution of any one of R319, H330, D334, H378, and H411.

18. The recombinant gram-negative bacterial host cell of any preceding claim, wherein the host cell is capable of high-density cell growth in culture.

19. The recombinant gram-negative bacterial host cell of claim 18, wherein the high-density cell growth in culture comprises growth to OD575 of about 80 to about 300.

20. The recombinant gram-negative bacterial host cell of claim 18 or 19, wherein the high-density cell growth in culture is increased in comparison to a control cell by about 2-fold to about 15-fold.

21. The recombinant gram-negative bacterial host cell of claim 20, wherein the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from:

(i) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional;

(ii) a recombinant gram-negative bacterial host cell deficient in the first protease activity, the second protease activity, and an additional protease activity as recited in 2(c), and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity of 2(c) that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and

(iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell.

22. The recombinant gram-negative bacterial host cell of claim 21, wherein the additional protease activity of 2(c) is an activity of a serralyisin precursor having the amino acid

sequence set forth as SEQ ID NO: 9; a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9.

23. The recombinant gram-negative bacterial host cell of any preceding claim, further comprising at least one expression construct, each expression construct comprising at least one nucleic acid sequence encoding a recombinant protein of interest.

24. The recombinant gram-negative bacterial host cell of claim 23, wherein the recombinant protein of interest is native or heterologous to the recombinant gram-negative bacterial host cell.

25. The recombinant gram-negative bacterial host cell of claim 23, wherein the recombinant protein of interest is selected from: an antibody, antibody fragment, or derivative of an antibody or antibody fragment; an antibody-based drug, a non-antibody binding protein (e.g., an antibody mimetic, including, but not limited to, an alphabody, an iBody, an affibody, an affilin, an affitin, or an anticalin), a reagent protein; a vaccine antigen; a therapeutic protein or enzyme; non-natural protein; a pathogen protein or derivative thereof; a microbial toxin, a lipoprotein; an extracellular receptor or ligand; a protease; a kinase; a blood protein; a chemokine; a cytokine; a bone morphogenic protein; an anticoagulant; a blood factor; a bone morphogenetic protein; an engineered protein scaffold; an enzyme, e.g., a biocatalytic enzyme; a growth factor; an interferon; an interleukin; a thrombolytic agent; a hormone; and a TGF-beta family member protein.

26. The recombinant gram-negative bacterial host cell of any one of claims 23-25, wherein the recombinant protein of interest is human, murine, rat, rabbit, guinea pig, camelid, shark, avian, yeast, fungal, gram-negative bacterial, or gram-positive bacterial.

27. The recombinant gram-negative bacterial host cell of claim 25 or 26, wherein the antibody, antibody fragment, or derivative thereof is selected from: a monoclonal antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; modified antibody, a bispecific antibody, a chimeric antibody; a diabody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a tribody; an intrabody; a nanobody; a small modular

immunopharmaceutical (SMIP); an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody, an avian antibody (e.g., a chicken antibody), a VHH-containing antibody; a F(ab); a F(ab)'; F(ab)'₂; scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment (e.g., generated by reducing the hinge region disulfide bonds of IgG); an Fc fusion protein (e.g., comprising the Fc domain of IgG fused together with a protein or peptide of interest); a domain antibody; a VL; a VNAR; a VH; and a VHH.

28. The recombinant gram-negative bacterial host cell of claim 27, wherein the VHH-containing antibody is a VHH concatenated antibody.

29. The recombinant gram-negative bacterial host cell of any one of claims 25 to 28, wherein the antibody, antibody fragment, or derivative thereof, binds to a target selected from: a cytokine; a chemokine; a drug; a cell-surface protein, e.g., a receptor, cell-surface marker, pathogen surface-protein, etc.; a growth factor; a growth factor receptor; immune checkpoint molecule, and a blood factor.

30. The recombinant gram-negative bacterial host cell of any one of claims 25 to 29, wherein the antibody, antibody fragment, or derivative thereof is a Fab'.

31. The recombinant gram-negative bacterial host cell of claim 30, wherein the Fab' binds to a target selected from: Carcinoembryonic antigen (CEA); CD22; fibrin II, beta chain; TNF-alpha; and NCA-90 (granulocyte antigen).

32. The recombinant gram-negative bacterial host cell of any one of claims 25 to 31, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least one nucleic acid sequence encoding a heavy chain, at least one nucleic acid sequence encoding a light chain, or both, wherein the heavy chain is full-length or a heavy chain fragment, and the light chain is full-length or a light chain fragment.

33. The recombinant gram-negative bacterial host cell of claim 32, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least two nucleic acid sequences, each encoding a heavy chain.

34. The recombinant gram-negative bacterial host cell of claim 32 or 33, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from the same mRNA transcript.

35. The recombinant gram-negative bacterial host cell of claim 32 or 33, wherein the at least one expression construct encoding the antibody, an antibody fragment, or derivative thereof comprises a nucleic acid sequence encoding a heavy chain and a nucleic acid sequence encoding a light chain, wherein the heavy and light chain are expressed from different mRNA transcripts.

36. The recombinant gram-negative bacterial host cell of claim 34 or 35, wherein each heavy chain-encoding nucleic acid sequence and each light chain-encoding nucleic acid sequence is individually operably linked to an independently selected nucleic acid sequence encoding a periplasmic secretion signal.

37. The recombinant gram-negative bacterial host cell of claim 36, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NO: 11, 13, 15, or 17.

38. The recombinant gram-negative bacterial host cell of any one of claims 34 to 37, wherein the expression construct comprises: a nucleic acid sequence encoding an antibody heavy chain, operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NOS: 11, 13, 15, or 17; a nucleic acid sequence encoding a light chain, operably linked to a nucleic acid sequence encoding a periplasmic secretion signal, wherein the periplasmic secretion signal has the amino acid sequence set forth as SEQ ID NOS: 11, 13, 15, or 17; or both.

39. The recombinant gram-negative bacterial host cell of any one of claims 25 to 38, wherein the antibody, antibody fragment, or derivative thereof is humanized.

40. The recombinant gram-negative bacterial host cell of any one of claims 30 to 39, wherein the Fab' is certolizumab.

41. The recombinant gram-negative bacterial host cell of claim 40, wherein the Fab' heavy chain has the amino acid sequence set forth as SEQ ID NO: 21, and the Fab' light chain has the amino acid sequence set forth as SEQ ID NO: 23.

42. The recombinant gram-negative bacterial host cell of any one of claims 32-41, wherein the nucleic acid sequence encoding the heavy chain is operably linked to a nucleic acid sequence encoding a secretion leader having the amino acid sequence set forth as SEQ ID NO: 11, and the nucleic acid sequence encoding the light chain is operably linked to a nucleic acid sequence encoding a secretion leader having the amino acid sequence set forth as SEQ ID NO: 13.

43. The recombinant gram-negative bacterial host cell of any one of claims 1-42, wherein the host cell is deficient in:

- (i) the first protease activity;
- (ii) the second protease activity;
- (iii) the activity of a serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9, a homologue of the serralyisin precursor having the amino acid sequence set forth as SEQ ID NO: 9, or a serralyisin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9;
- (iv) an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and
- (v) an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38.

44. The recombinant gram-negative bacterial host cell of claim 43, wherein the host cell further overexpresses an exogenous inactivated DegP, wherein the inactivated DegP is selected from: *P. fluorescens* DegP2 S219A; an inactivated DegP2 derived from the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from the amino acid sequence set forth as SEQ ID NO: 62; an inactivated DegP2 derived from a homologue of the amino acid sequence set forth as SEQ ID NO: 31; an inactivated DegP2 derived from a DegP2 having at least 60% similarity or at least 60% identity to amino acid sequence set forth as SEQ

ID NO: 31; an inactivated DegP2 derived from a DegP2 having at least 60% similarity or at least 60% identity to amino acid sequence set forth as SEQ ID NO: 62; and each of the proteases having the amino acid sequence set forth as SEQ ID NO: 31 comprising a conservative or nonconservative amino acid substitution or disruption of any one or more of positions: 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 146, 147, 148, 149, 150, 151, 152, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, and 234.

45. The recombinant gram-negative bacterial host cell of claim 43 or 44, wherein the host cell overexpresses an exogenous disulfide isomerase selected from any one of: a disulfide isomerase having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NO: 27, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, or 73, and a homologue of a disulfide isomerase having the amino acid sequence set forth as SEQ ID NO: 27, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, and 73.

46. The recombinant gram-negative bacterial host cell of any one of claims 1-45, wherein the recombinant gram-negative bacterial host cell is selected from: a *Pseudomonad* host cell; an *E. coli* host cell; and a *Vibrio* host cell.

47. The recombinant gram-negative bacterial host cell of claim 46, wherein the *Pseudomonad* host cell is a *Pseudomonas* host cell.

48. The recombinant gram-negative bacterial host cell of claim 47, wherein the *Pseudomonas* host cell is *P. fluorescens*, *P. putida*, or *P. aeruginosa*.

49. The recombinant gram-negative bacterial host cell of claim 47 or 48, wherein the cell is:

- (i) lsc::lacIQ1;
- (ii) Prc1-
- (ii) Prc2-
- (iii) HslU-
- (iv) HslV-
- (v) MepM1-
- (vi) PyrF-

and (vii) deficient in a serralysin precursor that is: a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; wherein the serralysin precursor deficiency results from a mutation in a gene encoding the serralysin precursor.

50. The recombinant gram-negative bacterial host cell of claim 49, wherein the cell is *P. fluorescens*, the Prc1 has the amino acid sequence set forth in SEQ ID NO: 33, the Prc2 has the amino acid sequence set forth in (SEQ ID NO: 35), the HslU has the amino acid sequence set forth in (SEQ ID NO: 37), the HslV has the amino acid sequence set forth in (SEQ ID NO: 38), the MepM1 has the amino acid sequence set forth in (SEQ ID NO: 1), and the serralysin precursor has the amino acid sequence set forth as SEQ ID NO: 9.

51. The recombinant gram-negative bacterial host cell of claim 50, further comprising an expression vector comprising a nucleic acid sequence encoding DegP2 S219A (SEQ ID NO: 29).

52. The recombinant gram-negative bacterial host cell of claim 50 or 51, further comprising an expression vector comprising a nucleic acid sequence encoding disulfide isomerase PDIA6 (SEQ ID NO: 27).

53. The recombinant gram-negative bacterial host cell of any one of claims 1-52, further comprising an expression vector encoding a recombinant protein.

54. The recombinant gram-negative bacterial host cell of claim 53, wherein the expression vector encodes a Fab'.

55. The recombinant gram-negative bacterial host cell of claim 54, wherein the expression vector comprising the nucleic acid sequence encoding DegP2 S219A or disulfide isomerase PDIA6 further comprises a nucleic acid sequence encoding the Fab'.

56. The recombinant gram-negative bacterial host cell of claim 54 or 55, wherein the Fab' heavy chain is encoded by SEQ ID NO: 21, and the Fab' light chain is encoded by SEQ ID NO: 23.

57. The recombinant gram-negative bacterial host cell of claim 1, wherein the recombinant gram-negative bacterial host cell is a *Pseudomonad* having the genotype of strain STR94975, STR94976, or STR94977.

58. The recombinant gram-negative bacterial host cell of claim 57, further comprising the expression construct or constructs of STR94975, STR94976, or STR94977, for use in producing a recombinant anti-TNF-alpha Fab'.

59. A method for producing a recombinant protein of interest comprising: (a) recovering the recombinant protein of interest from a recombinant gram-negative bacterial host cell of any one of claims 1-57 cultured under suitable fermentation conditions, wherein the recombinant gram-negative host cell is transformed with a plasmid comprising a nucleic acid encoding the recombinant protein of interest.

60. The method of claim 59, wherein transcription of the nucleic acid sequence encoding the recombinant protein of interest is regulated by an inducible promoter.

61. The method of claim 60, wherein the inducible promoter is selected from: a tac promoter, a mannitol promoter, a Pben, a T7 promoter, a lac promoter, a T5 promoter, a xylose promoter, and an arabinose promoter.

62. The method of any one of claims 59-61, wherein the recombinant gram-negative bacterial host cell can grow to high cell density.

63. The method of claim 62, wherein the high cell density comprises an OD575 of about 80 to about 300.

64. The method of any one of claims 58-63, wherein the suitable fermentation conditions comprise induction of the inducible promoter at: an OD575 of about 80 to about 160, a culture pH of about 5.8 to about 7.0, a temperature of about 28-33 deg C, fed-batch, and a titer range of about 0.2 to about 5 g/L.

65. The method of claim 64, wherein the inducible promoter is induced by IPTG, and wherein the IPTG is added to a final concentration of about 0.08 – 0.3mM.

66. The method of claim 65, wherein the IPTG is added to a final concentration of about 0.2 mM.

67. The method of any one of claims 63-66, wherein induction is carried out at a culture pH of about 6.0 to about 6.5.

68. The method of any one of claims 63-67, wherein induction is carried out at a temperature of about 28-33 deg C.

69. The method of claim 68, wherein induction is carried out at a temperature of about 32 deg C.

70. The method of any one of claims 59-69, wherein the recombinant gram-negative bacterial host cell grows to a cell density that is increased in comparison to a control cell grown under the same fermentation conditions.

71. The method of claim 70, wherein the increase in cell density is about 2-fold to about 15-fold.

72. The method of any one of claims 59-71, further comprising: (b) measuring the yield of intact, soluble, and/or active, recombinant protein of interest recovered from the recombinant gram-negative bacterial host cell.

73. The method of claim 72, wherein the measured yield of intact, soluble, and/or active, recombinant protein is about 0.1 to about 10 g/L.

74. The method of claim 72 or 73, further comprising: (c) measuring the yield of recombinant protein of interest recovered from a control cell that is intact, soluble, active, or a combination thereof.

75. The method of claim 74, further comprising (d) comparing the yield measured in step (b) to the yield measured in step (c).

76. The method of claim 75, wherein the yield measured in step (b) is about 2-fold to about 100-fold higher than that measured in step (c).

77. The method of any one of claims 70-76, wherein the recombinant gram-negative bacterial host cell and the control cell, respectively, are selected from:

(i) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and a corresponding gram-negative bacterial host cell deficient in the first protease activity and wherein the second protease is functional;

(ii) a recombinant gram-negative bacterial host cell deficient in the first protease activity, the second protease activity, and an additional protease activity as recited in 2(a), and a corresponding gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and wherein the additional protease activity of 2(a) that is deficient in the compared recombinant gram-negative bacterial host cell is functional; and

(iii) a recombinant gram-negative bacterial host cell deficient in the first protease activity and the second protease activity, and comprising a functional protease that is: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; a homologue of the MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 amino acid sequence set forth as SEQ ID NO: 5, and a corresponding gram-negative bacterial host cell deficient in the activity of the first protease and the second protease, and deficient in the functional protease of the compared recombinant gram-negative bacterial host cell.

78. The recombinant gram-negative bacterial host cell of any one of claims 1-45, or any one of claims 47-77, wherein the gram-negative bacterial host cell is not *E. coli*.

79. A recombinant polypeptide comprising: a secretion signal peptide operably linked to a heterologous protein or polypeptide of interest, wherein the secretion signal peptide has the amino acid sequence set forth in SEQ ID NO: 11.

80. The polypeptide of claim 79, wherein the protein or polypeptide of interest is selected from: an antibody, antibody fragment, or a derivative of an antibody or an antibody fragment; an enzyme; a cytokine; a chemokine; a growth factor; a fusion protein; and a vaccine antigen.

81. The polypeptide of claim 79 or 80, wherein the antibody, antibody fragment, or a derivative of an antibody or antibody fragment is selected from: a monoclonal antibody; a full chain antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; a modified antibody; a variable-region only antibody fragment; a bispecific antibody, a chimeric antibody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a dibody; an intrabody; a nanobody; a small modular immunopharmaceutical; an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody (VNAR); an avian antibody; a VHH; a VHH-containing antibody; a VHH concatemer; a F(ab); a F(ab)'; F(ab)'₂; an scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment; an Fc fusion protein; a domain antibody; a VL; and a VH.

82. The polypeptide of claim 80 or 81, wherein the antibody, antibody fragment, or derivative of the antibody or antibody fragment is humanized.

83. The polypeptide of claim 80, wherein the enzyme is a therapeutic enzyme.

84. The polypeptide of claim 83, wherein the therapeutic enzyme is selected from: a peptidase; a lactase; an amylase; a PEP; a digestive enzyme; a uricase; a rhodanase; a urokinase; a streptokinase; a staphylokinase; a phenylase; a sacrosidase; a lysozyme; a chitinase; a ribonuclease; a glutaminase; an arginase; a vibrilase; a chondroitinase; a hyaluronidase; a galactosidase; a glucuronidase; a glucocerebrosidase; a thymidine phosphorylase; a carbonic anhydrase; a uricase thiosulfate-cyanide; a sulfurtransferase; a phosphothioesterase; an alcohol oxidase; an alcohol dehydrogenase; an asparaginase; a glutamine synthase; an adenosine deaminase; bovine pegademase; alglucerase; dornase alpha; imiglucerase; sacrosidase; rasburicase; agalsidase beta; and nattokinase.

85. The polypeptide of claim 80, wherein the fusion protein is selected from: an enzyme fusion protein; a protein A fusion protein; an albumin fusion protein; a thioredoxin fusion protein; a ubiquitin fusion protein; a streptavidin fusion protein; a maltose binding protein fusion

protein; a chitin being protein fusion protein; a SUMO fusion protein; and a glutathione-S-transferase fusion protein.

86. The polypeptide of any one of claims 79-85, further comprising a linker.

87. The polypeptide of any one of claims 79-86, further comprising a cleavage domain.

88. The polypeptide of any one of claims 79-87, wherein the secretion signal peptide directs expression of the protein or polypeptide of interest to the periplasm or the extracellular space of a prokaryotic host cell.

89. The polypeptide of claim 88, wherein the prokaryotic host cell is a gram-negative bacterium.

90. The polypeptide of claim 88, wherein the prokaryotic host cell is a gram-positive bacterium.

91. The polypeptide of claim 89, wherein the gram-negative bacterium is a *Pseudomonad*, *V. natriegens*, or *E. coli*.

92. The polypeptide of claim 90, wherein the gram-positive bacterium is a *Corynebacterium* or a *Bacillus*.

93. A method of producing a protein or polypeptide of interest in a prokaryotic host cell, the method comprising: producing the protein or polypeptide of interest in the periplasm of a prokaryotic host cell cultured in a cell culture growth medium, wherein the prokaryotic host cell comprises an expression construct comprising a nucleic acid encoding a recombinant polypeptide comprising the protein or polypeptide of interest operably linked to a secretion signal peptide that directs expression of the protein or polypeptide of interest to the periplasm of the prokaryotic host cell, wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 11, and wherein the secretion signal peptide is not native to the protein or polypeptide of interest.

94. The method of claim 93, further comprising isolating the produced protein or polypeptide of interest.

95. The method of claim 94, wherein the protein or polypeptide of interest is selected from: an antibody, antibody fragment, or a derivative of an antibody or an antibody fragment; an enzyme; a cytokine; a chemokine; a growth factor; a fusion protein; and a vaccine antigen.

96. The method of claim 95, wherein the antibody, antibody fragment, or a derivative of an antibody or an antibody fragment is selected from: a monoclonal antibody; a full chain antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; a modified antibody; a variable-region only antibody fragment; a bispecific antibody, a chimeric antibody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a dibody; an intrabody; a nanobody; a small modular immunopharmaceutical; an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody (VNAR); an avian antibody; a VHH; a VHH-containing antibody; a VHH concatemer; a F(ab); a F(ab)'; F(ab)'₂; an scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment; an Fc fusion protein; a domain antibody; a VL; and a VH.

97. The method of claim 95 or 96, wherein the antibody, antibody fragment, or derivative of the antibody or antibody fragment is humanized.

98. The method of claim 95, wherein the enzyme is a therapeutic enzyme.

99. The method of claim 98, wherein the therapeutic enzyme is selected from: a peptidase; a lactase; an amylase; a PEP; a digestive enzyme; a uricase; a rhodanase; a urokinase; a streptokinase; a staphylokinase; a phenylase; a sacrosidase; a lysozyme; a chitinase; a ribonuclease; a glutaminase; an arginase; a vibrilase; a chondroitinase; a hyaluronidase; a galactosidase; a glucuronidase; a glucocerebrosidase; a thymidine phosphorylase; a carbonic anhydrase; a uricase thiosulfate-cyanide; a sulfurtransferase; a phosphothioesterase; an alcohol oxidase; an alcohol dehydrogenase; an asparaginase; a glutamine synthase; an adenosine deaminase; bovine pegademase; alglucerase; dornase alpha; imiglucerase; sacrosidase; rasburicase; agalsidase beta; and nattokinase.

100. The method of claim 95, wherein the fusion protein is selected from: an enzyme fusion protein; a protein A fusion protein; an albumin fusion protein; a thioredoxin fusion protein; a ubiquitin fusion protein; a streptavidin fusion protein; a maltose binding protein fusion

protein; a chitin being protein fusion protein; a SUMO fusion protein; and a glutathione-S-transferase fusion protein.

101. The method of any one of claims 93-100, wherein the nucleic acid encodes a linker.

102. The method of claim 101, wherein the linker comprises a cleavage domain.

103. The method of any one of claims 93-102, wherein the prokaryotic host cell is a gram-negative bacterium.

104. The method of any one of claims 93-102, wherein the prokaryotic host cell is a gram-positive bacterium.

105. The method of claim 93, wherein the gram-negative bacterium is a *Pseudomonad*, *V. natriegens*, or *E. coli*.

106. The method of claim 94, wherein the gram-positive bacterium is a *Corynebacterium* or a *Bacillus*.

107. An expression vector comprising a nucleic acid sequence encoding a recombinant polypeptide of any one of claims 79-92.

108. A prokaryotic host cell comprising an expression vector of claim 107.

109. The prokaryotic host cell of claim 108, wherein the prokaryotic host cell is a gram-negative bacterium.

110. The prokaryotic host cell of claim 108, wherein the prokaryotic host cell is a gram-positive bacterium.

111. The prokaryotic host cell of claim 109, wherein the gram-negative bacterium is a *Pseudomonad*, *V. natriegens*, or *E. coli*.

112. The prokaryotic host cell of claim 110, wherein the gram-positive bacterium is a *Corynebacterium* or a *Bacillus*.

113. The prokaryotic host cell of any one of claims 108-112, wherein the nucleic acid sequence encoding the recombinant polypeptide is optimized for expression in the prokaryotic host cell.

114. Use of a recombinant polypeptide of any one of claims 79-92, an expression vector of claim 107, or a prokaryotic host cell of any one of claims 108-113, for expressing a protein or polypeptide of interest in the periplasm or the extracellular space of a prokaryotic host cell.